Transpl Int (1997) 10: 293–298

ORIGINAL ARTICLE

Kimikazu Hamano
Hiroshi Ito
Andrew Bushell
Kathryn J. Wood
Kensuke Esato

The induction of operational tolerance is not prevented by simultaneous administration of cyclosporin A[1]

Received: 2 October 1996
Received after revision: 31 January 1997
Accepted: 5 February 1997

K. Hamano (✉) · H. Ito · K. Esato
First Department of Surgery,
Yamaguchi University, School of Medicine,
1144 Kogushi, Ube,
Yamaguchi, 755, Japan
Fax: +81 836 22 22 60

A. Bushell · K.J. Wood
Nuffield Department of Surgery,
University of Oxford,
John Radcliffe Hospital,
Headington, Oxford OX3 9DU, UK

Abstract In this study, the effect of combining anti-CD4 monoclonal antibody (mAb) and cyclosporin (CyA) therapy at the time of transplantation was examined. A mouse cardiac allograft model was used. Anti-CD4 mAb administered perioperatively induces long-term survival. The addition of a short course of CyA given subcutaneously in a regimen of either a high-dose treatment or a standard dose treatment to the anti-CD4 mAb treatment protocol did not have a detrimental effect on graft survival. Despite having no significant effect on graft survival, the addition of CyA to the treatment protocol did result in a significant decrease in the level of IL-2 present in the hearts 7 days after transplantation. The decrease in IL-2 production was directly related to the presence of CyA in vivo. When CyA treatment was continued throughout the period during which unresponsiveness to the graft is induced by anti-CD4 mAb therapy, 50 % of the grafted hearts were rejected once the CyA was discontinued. In conclusion, the combined use of anti-CD4 mAb therapy and CyA did not have a negative effect on graft survival in this model when the two agents were used concurrently at the time of transplantation.

Key words Cyclosporin, heart transplantation, mouse · Heart transplantation, cyclosporin, mouse · Tolerance, anti-CD4, cyclosporin · Anti-CD4, cyclosporin, tolerance

Introduction

Monoclonal antibodies specific for leukocyte cell surface molecules have been shown to have beneficial effects on graft survival [5, 7, 10, 13]. Anti-CD4 monoclonal antibodies (mAb) have been shown to be potent immunosuppressive agents in a variety of model systems. In addition, they may have the ability to induce specific unresponsiveness to the graft in some situations. In the majority of experimental studies anti-CD4 mAbs have been used as the sole immunosuppressive agent. This is unlikely to be the case when these agents are introduced into clinical transplantation. If anti-CD4 mAbs are to be used widely in clinical transplantation, they will most probably be used as additional therapy. Therefore they will be administered in combination with conventional immunosuppressive agents, such as cyclosporin (CyA). Anti-CD4 and CyA suppress the rejection response, but have different mechanisms of action [8, 21]. It is therefore very important to investigate the outcome of combining anti-CD4 mAbs with conventional immunosuppressive agents to evaluate whether there are any positive and/or negative effects on the immunosuppressive properties of either agent. In this study, we have used a mouse heart transplantation model to investigate the effect of the combined concurrent treatment with anti-CD4 mAb and CyA at the time of transplantation.

Materials and methods

Mice

C3H/He (H-2^k) and C57BL/10 (H-2^b) were obtained from Chiyoda Kaihatsu (Japan) and housed under clean conditions at the Institute of Laboratory Animals, Yamaguchi University, School of Medicine. Mice between 8 and 12 weeks old were used for all experiments. This experiment was reviewed by the Committee of the Ethics on Animal Experiments of the Yamaguchi University School of Medicine and was carried out under the control of the Guidelines for Animal Experiments of the Yamaguchi University School of Medicine, Law No. 105 and Notification (No. 6) of the government. All animals have received humane care in compliance with the *Principles of laboratory animal care* (NIH Publication No. 86–23, revised 1985).

Heart transplantation

Heterotopic heart transplantation was performed in the abdominal cavity [6]. C3H/He mice received C57BL/6 hearts. Graft survival was monitored by daily palpation of the graft and electrocardiograms as required [24].

Anti-CD4 monoclonal antibody

YTA3.1.2 (Rat anti mouse CD4 monoclonal antibody, IgG2b) [19] was kindly provided by Professor H. Waldmann (Oxford). YTA3.1.2 was taken from the ascites and purified as described previously [16]. Fifty micrograms of YTA3.1.2 was administered intravenously (i.v.) the day before and the day of transplantation [16].

Administration of cyclosporin

To evaluate the effect of the combined treatment of anti-CD4 mAb and CyA, CyA was administered in two treatment regimens: (1) a high-dose regimen – 100 mg/kg per day of CyA was administered subcutaneously (sc) on days 0, 4, and 6 after transplantation [14] and (2) a standard dose regimen – 15 mg/kg per day of CyA was administered sc for 7 days after transplantation.

Anti-CD4 mAb alone has been shown to induce specific unresponsiveness to donor alloantigens present on the transplanted heart by 50 days after transplantation [9]. In the third part of this study we therefore continued to administer CyA (10 mg/kg per day, sc) throughout this induction period.

Measurement of cytokine expression in the transplanted hearts

The grafted hearts were harvested, and homogenized with 600 µl of phosphate-buffered saline (PBS). The samples were then centrifuged at 10000 *g* for 10 min at 4 °C, and the supernatant was used for the cytokine measurement. IL-2 levels were measured with the Inter Test-2X™ Mouse IL-2 ELISA Kit (Code no. 212201; Genzyme, USA) [11]. The protein content of each sample was determined with the Coomassie Brilliant Blue G-250 method and the Tonein-TP assay (Otuka Pharmaceutica, Japan) [20]. IL-2 levels in the grafted hearts are reported as IL-2/mg protein.

Statistical analysis

Allograft survival among groups within an experiment was compared by the Kruskal-Wallis test, and two groups were compared to each other with the Mann-Whitney U-test. The IL-2 levels present in the grafted hearts were also compared with the Mann-Whitney U-test.

Results

Graft survival (Fig. 1)

High-dose CyA treatment regimen

Untreated C3H/He mice rejected C57BL/6 hearts within 8 days after transplantation ($n = 5$). Administration of a depleting anti-CD4 mAb, YTA3.1.2, the day before and the day of transplantation prolonged graft survival; median graft survival time (MST 72 days, range 32 days to > 100 days, $n = 6$; $P = 0.006$). Treatment with a high-dose of CyA (100 mg/kg) on days 0, 4, 6 after transplantation also prolonged graft survival, but was much less effective than anti-CD4 mAb in this model (MST 16 days, $n = 5$; $P = 0.009$). The combined treatment of YTA3.1.2 and high-dose CyA did not destroy the ability of the anti-CD4 mAb therapy to prolong graft survival, but interestingly, the concurrent therapy did not have any beneficial effect either (MST 77 days, range 14 days to > 100 days, $n = 6$).

Standard dose CyA treatment regimen

Treatment with CyA (15 mg/kg per day sc) for 7 days after transplantation also had a small effect on graft survival compared to untreated controls (MST 15 days, $n = 6$; $P = 0.006$). The combined treatment with YTA3.1.2 and CyA at this dosage did not inhibit the ability of anti-CD4 mAb therapy to prolong graft survival (MST 76 days, range 18 days to > 100 days, $n = 6$).

Cytokine levels in the transplanted hearts

IL-2 levels present in the transplanted hearts were measured for each treatment protocol 7 days after transplantation (Fig. 2a). In untreated mice with beating hearts 7 days after transplantation, 9.51 ± 1.07 pg IL-2/mg protein was detected within the transplanted heart ($n = 5$). Interestingly, the amount of IL-2 present in the transplanted hearts of mice receiving anti-CD4 mAb therapy was not significantly different from that found in hearts being rejected at this point in time: 9.42 ± 2.63 pg IL-2/mg protein ($n = 5$). CyA therapy alone reduced the amount of IL-2 detectable within the hearts to 7.29 ± 0.83 pg IL-2/mg protein ($n = 5$) even though

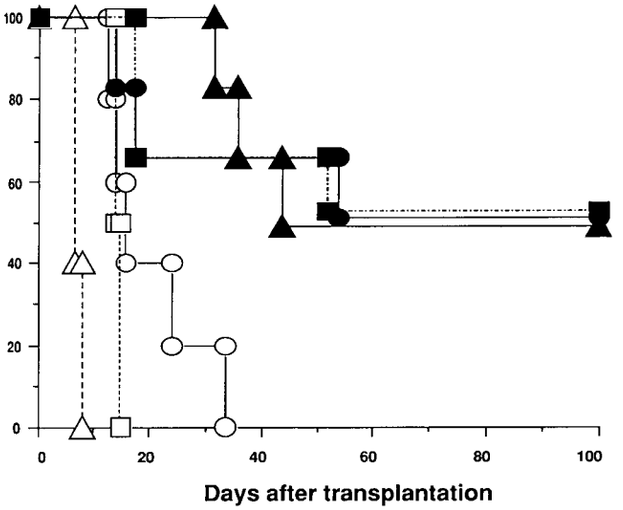

Fig. 1 Graft survival of the combined treatment of anti-CD4 mAb and a short course of CyA. C57BL/6 hearts were transplanted into the abdominal cavity of the C3H/He mice. All C57BL/6 hearts were rejected within 8 days after transplantation without treatment (--△--, MST = 7 days, $n = 5$). Anti-CD4 mAb (YTA3.1.2) treatment prolonged the graft survival (—▲—, MST = 72 days, $n = 6$). Standard dose CyA treatment (15 mg/kg per day for 7 days after transplantation; ---□---, MST = 15 days, $n = 6$) or high-dose CyA treatment (100 mg/kg per day, days 0, 4 and 6; ---○---, MST = 16 days, $n = 5$) prolonged the graft survival slightly. The combined treatment of anti-CD4 mAb and either high-dose (---●---, MST = 77 days, $n = 6$) or standard dose CyA(---■---, MST = 76 days, $n = 6$) did not significantly prolong the graft survival compared with anti-CD4 mAb treatment alone

median graft survival in this group was only 16 days. Combined treatment with both YTA3.1.2 and high-dose CyA reduced the amount of IL-2 detectable within the heart significantly compared to either treatment alone: 5.09 ± 0.48 pg IL-2/mg protein ($n = 5$).

To determine if the reduction in IL-2 levels in the presence of CyA was maintained after therapy was discontinued, IL-2 levels were examined 21 days after transplantation in the mice treated with YTA3.1.2 alone or combined YTA3.1.2 and high-dose CyA (Fig. 2b). The IL-2 levels present in the group receiving anti-CD4 mAb therapy alone or the combined treatment were not significantly different: 4.28 ± 1.36 and 4.59 ± 2.61 pg IL-2/mg protein, respectively.

Continuous administration of CyA during the induction of unresponsiveness

Previously, we reported that perioperative treatment with YTA3.1.2 can induce specific unresponsiveness to the donor alloantigens by 50 days after transplantation [9]. We therefore examined whether continuous administration of CyA (10 mg/kg per day, sc) during this induction period would adversely affect the development of unresponsiveness when treatment was combined with anti-CD4 mAb. During CyA treatment, all grafted hearts continued to function in both groups. In recipients receiving CyA therapy alone, the transplanted hearts ceased to be rejected within 12 days after CyA treatment (MST = 60 days, $n = 5$). When YTA3.1.2 and long-term CyA therapy were combined, graft survival was unexpectedly not prolonged in all recipients; three out of six grafts were rejected when CyA therapy was stopped (MST = 90 days, $n = 6$; Fig. 3).

Discussion

Anti-CD4 mAbs are currently being clinically evaluated to determine their efficacy in the prevention of allograft rejection. In experimental studies, anti-CD4 mAbs have been shown to be potent immunosuppressive agents in a number of models [5, 7, 8, 13]. They have been shown to possess the ability to induce antigen-specific unresponsiveness to alloantigens in vivo when used as the sole immunosuppressive agent [9, 16, 19]. This latter property would offer many advantages if it could be reproduced in the clinical environment. However, at present it is not clear whether the immunosuppressive properties of anti-CD4 mAbs are affected by the presence of the immunosuppressive drugs currently used in clinical transplantation. As it is unlikely that anti-CD4 mAbs will be used as the only form of immunosuppressive therapy in the clinical situation, we investigated whether the addition of CyA, either as a short course or long-term therapy, would affect the immunosuppressive properties of anti-CD4 used at the time of transplantation.

The mechanisms responsible for suppression of immune responses by either anti-CD4 mAb therapy or CyA are likely to be distinct. Anti-CD4 mAbs are thought to act by blocking the effective delivery of signals to the T cell at the time of antigen recognition by preventing the interaction between the CD4 molecule and major histocompatibility complex (MHC) class II-peptide complex on the antigen-presenting cell [8]. In contrast, CyA interferes with induction of cytokine genes [22]. CyA binds to its cytoplasmic receptor, cyclophilin, and this CyA-cyclophilin complex leads to binding and inactivation of calcineurin B, a major cellular phosphatase [2], resulting in a reduction in the availability of the transcription factor NF [22]. CyA inhibition of T-cell signal transduction renders the helper T cell unable to initiate an immune response by either the production of lymphokines or by proliferation [2]. In contrast, the induction of specific unresponsiveness by anti-CD4 mAbs is thought to be due to their ability to activate Th2 cells [1, 23]. Taken together, these findings suggest that the addition of CyA to protocols using anti-CD4 mAbs may prevent their ability to induce tolerance.

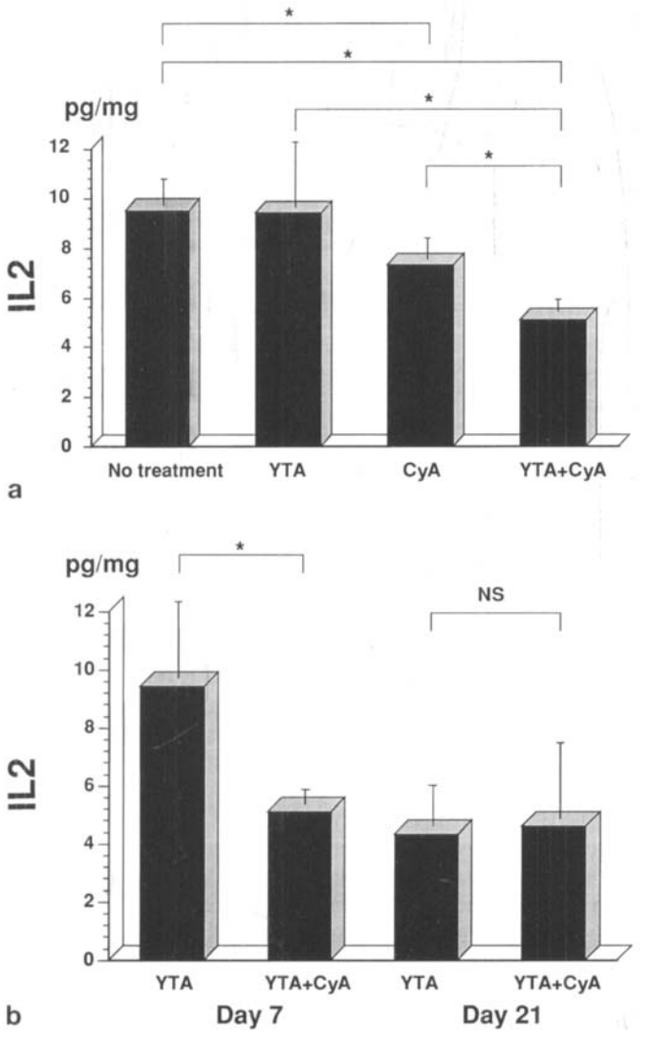

Fig. 2a IL-2 levels present in the transplanted hearts 7 days after transplantation. IL-2 levels in the hearts of untreated ($n = 5$) and anti-CD4 mAb-treated recipients (YTA; $n = 5$) were 9.51 ± 1.07 and 9.42 ± 2.63 pg/mg protein, respectively. There was no significant difference between the two groups. IL-2 levels in the high-dose CyA group (CyA; $n = 5$; 7.29 ± 0.83 pg/mg protein) were reduced significantly compared with the untreated control. The combined treatment of anti-CD4 mAb and high-dose CyA (YTA + CyA; $n = 5$; 5.09 ± 0.48) reduced IL-2 levels significantly compared with either treatment alone. **b** IL-2 levels present in the transplanted hearts in anti-CD4 mAb treatment (YTA) and the combined treatment of anti-CD4 mAb and high-dose CyA (YTA + CyA) 7 and 21 days after transplantation. IL-2 levels in the heart grafts of mice treated with anti-CD4 mAb and high-dose CyA was significantly suppressed compared with that in the hearts of mice receiving anti-CD4 mAb treatment alone 7 days after transplantation ($P = 0.0068$). However, by 21 days after transplantation, there was no significant difference in IL-2 levels between the YTA group and the YTA + CyA group

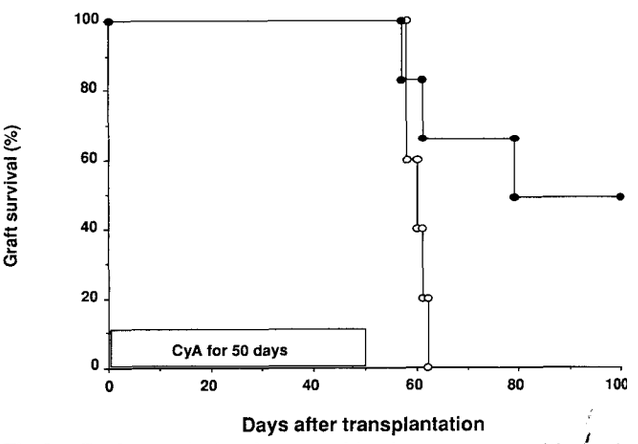

Fig. 3 Graft survival after combined treatment with anti-CD4 mAb and a prolonged course of CyA. Anti-CD4 mAb (YTA3.1.2) was given i.v. on the day before and the day of transplantation. A total of 10 mg/kg per day of CyA was given, subcutaneously, daily for 50 days after transplantation. In the CyA treatment group (—○—), all hearts were rejected 12 days after CyA administration ended (MST = 60 days, $n = 5$). In the combined treatment group, YTA3.1.2 and CyA (10 mg/kg per day for 50 days after transplantation) (—●—), three out of six grafts were rejected during the first 100 days after transplantation (MST = 90 days, $n = 6$)

The findings from our study show that CyA did not inhibit the immunosuppressive effects of anti-CD4 mAb therapy when given as a short course at the time of transplantation. As expected, the addition of CyA to the protocol inhibited the production of IL-2 by graft infiltrating cells within the transplanted hearts. However, the levels of IL-2 in the grafted hearts were similar in both groups after CyA had been cleared from the systemic circulation. CyA levels in the blood were measured 7 days after the final administration in both the high-dose and the standard dose treatments. CyA was at background level in both treatment groups (data not shown). The effect of the addition of CyA on the production of other cytokines by graft infiltrating cells is currently under investigation.

Previous results obtained in this mouse model have indicated that, following the perioperative administration of YTA 3.1.2 operational tolerance does not develop until about 30 days post-transplant [17]. Therefore, we investigated the long-term interaction between CyA and anti-CD4 mAb. When CyA was administered continuously for 50 days after transplantation, to cover the time needed for the induction of the unresponsive state, there was an adverse effect on long-term graft survival with 50 % of the three out of six mice rejecting their grafts once CyA was discontinued. Since graft survival in this combined protocol at 100 days was almost identical to that obtained with anti-CD4 mAb only (Fig. 1), we conclude that anti-CD4 mAb and CyA, in this model at

least, have no additive effect. The reasons for this lack of effect are not known at present. However, one possibility is that, although they have different modes of action, CyA and anti-CD4 mAb probably have overlapping effects on the T cells most closely involved in allorecognition and graft rejection. Anti-CD4 mAb alone clearly leads to tolerance in at least 50% of the recipients (Fig. 1). Although CyA would target most if not all, of the alloreactive T cells in the recipients, if these are already tolerant as a result of the antibody treatment, then CyA might not be able to exert any additional effect. Since tolerance induced by anti-CD4 mAb therapy appears to be an active process involving CD4 T-cell regulation [3, 4, 18], it is possible that CyA could have a negative effect on the action of anti-CD4 mAb. Our data, however, indicate that this is not the case.

There are conflicting results from other studies where biological agents that target T-cell and antigen-presenting cell function have been used together with CyA. For example, several studies have examined the effect of combining CyA with other agents such as mabs, e.g. anti-IL2R [25–27], or soluble recombinant ligands, e.g., CTLA4-Ig [2]. However, other experimental studies have shown that CyA can inhibit the beneficial effects of biological agents such as CTLA4-Ig and anti-CD40 ligand when the three agents are used simultaneously [12]. Indeed, cessation of CyA therapy resulted in skin graft rejection in this model; a phenomenon in keeping with the data obtained in this study (Fig. 3). Data from clinical renal transplantation where CyA has been used in combination with the monoclonal antibody OKT3 adicates a sequential, rather than simultaneous, administration of the biological agent and CyA may be advantageous [15]. Much more information is required before a clear understanding of how biological immunosuppressive agents and CyA can or should be combined. At the time of the present study, we note that the effects of prolonged CyA therapy combined with anti-CD4 mAb treatment have not been examined previously. Combined treatment with anti-CD4 mAb and a short course of CyA at the time of transplantation did not result in an additive immunosuppressive effect. More importantly, however, CyA did not prevent tolerance induction by anti-CD4 mAb when used either at the time of transplant or continuously for 50 days post-transplant. Our data indicate that the effects of anti-CD4 mAb and CyA are independent of each other. They also suggest that the potential benefit of anti-CD4 mAb therapy will not be lost if these reagents are incorporated into conventional clinical immunosuppressive protocol.

Acknowledgements. This work was supported by the Scientific Research Fund (No. 07771043) of the Ministry of Education in Japan and the British Heart Foundation.

References

1. Alters SE, Shizuru JA, Ackerman J, Grossman D, Seydel KB, Fathman CG (1991) Anti-CD4 mediates clonal anergy during transplantation tolerance induction. J Exp Med 173: 491–494
2. Bolling SF, Lin H, Wei R, Linsley P, Turka L (1994) The effect of combination cyclosporine and CTLA4-Ig therapy on cardiac allograft survival. J Surg Res 57: 60–64
3. Bushell A, Morris PJ, Wood KJ (1995) Transplantation tolerance induced by antigen pretreatment and depleting anti-CD4 antibody depends on CD4 + T cell regulation during the induction phase of the response. Eur J Immunol 25: 2643–2649
4. Chen ZK, Cobbold SP, Waldmann H, Metcalfe S (1996) Amplification of natural regulatory immune mechanisms for transplantation tolerance. Transplantation 62: 1200–1206
5. Cobbold S, Waldmann H (1986) Skin allograft rejection by L3T4$^+$ and LYT-2$^+$ T cell subset. Transplantation 41: 634–639
6. Corry RJ, Winn HJ, Russell PS (1973) Primarily vascularized allografts of hearts in mice: the role of H-2D, H-2 K, and non H-2 antigens in rejection. Transplantation 16: 343–350
7. Cosimi AB, Delmonico FL, Wright KJ, Wee S, Preffer FI, Jolliffe LK, Colvin RB (1990) Prolonged survival of non-human primate renal allograft recipients treated with only anti-CD4 monoclonal antibody. Surgery 108: 406–414
8. Darby CR, Morris PJ, Wood KJ (1992) Evidence that long-term cardiac allograft survival induced by anti-CD4 monoclonal antibody does not require depletion of CD4$^+$ T cells. Transplantation 54: 483–490
9. Hamano K, Rawsthorne M, Bushell A, Morris PJ, Wood KJ (1995) Donor microchimerism is not responsible for the maintenance of tolerance to donor alloantigens in recipients tolerant of cardiac allografts. Transplant Proc 27: 151
10. Isobe M, Yagita H, Okumura K (1992) Specific acceptance of cardiac allografts after treatment with antibodies to ICAM-1 and LFA-1. Science 255: 1125–1127
11. Kuziel WA, Greene WC (1990) Interleukin-2 and the IL-2 receptor: new insights into structure and function. J Invest Dermatol 94: 27S-32S
12. Larsen CP, Elwood ET, Alexander DZ, Ritchie SC, Hendrix R, Tucker-Burden C, Cho HR, Aruffo A, Hollenbaugh D, Linsley PS, Winn KJ, Pearson TC (1996) Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. Nature 381: 434–438
13. Madsen JC, Peugh WN, Wood KJ, Morris PJ (1987) The effect of anti-L3T4 monoclonal antibody treatment on first set rejection of murine cardiac allografts. Transplantation 44: 849–852
14. Mottram PL, Mirisklavos A, Dumble LJ, Clunie GJA (1990) T suppressor cells induced by transfusions and cyclosporine. Studies in the murine cardiac allograft model. Transplantation 50: 1033–1037
15. Opelz G (1995) Efficacy of rejection prophylaxis with OKT3 in renal transplantation. Collaborative Transplant Study. Transplantation 60: 1220–1224

16. Pearson TC, Madsen JC, Larsen CP, Morris PJ, Wood KJ (1992) Induction of transplantation tolerance in adults using donor antigen and anti-CD4 monoclonal antibody. Transplantation 54: 475–483
17. Pearson TC, Darby CR, Bushell AR, Morris PJ, Wood KJ (1993) The assessment of transplantation tolerance induced by anti-CD4 monoclonal antibody in the murine model. Transplantation 55: 361–367
18. Qin S, Cobbold S, Tighe H, Benjamin R, Waldmann H (1987) CD4 monoclonal antibody pairs for immunosuppression and tolerance induction. Eur J Immunol 17: 1159–1165
19. Qin S, Cobbold SP, Pope H, Elliott J, Kioussis D, Davies J, Waldmann H (1993) "Infectious" transplantation tolerance. Science 259: 974–977
20. Sano Shiba K, Kanaamori K, Harada T, Nakao M, Nakajima K, Kodaira T, Nakagawa H (1985) A cause of discrepancy between values for urinary protein as assayed by the coomassie brilliant blue G-250 method and the sulfosalicylic acid method. Clin Chem 31: 1215–1218
21. Schreiber SL, Crabtree GR (1992) The mechanism of action of cyclosporin A and FK506. Immunol Today 13: 136–142
22. Schwartz RH (1992) Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. Cell 71: 1065–1068
23. Stumbles P, Mason D (1995) Activation of $CD4^+$ T cells in the presence of a non depleting monoclonal antibody to CD4 induces a Th2-type response in vitro. J Exp Med 182: 5–13
24. Superina RA, Peugh WN, Wood KJ, Morris PJ (1986) Assessment of primarily vascularised cardiac allografts in mice. Transplantation 42: 226–227
25. Tilney NL, Padberg WM, Lord RHH, Araneda D, Strom TB, Kupiec-Weglinski JW (1988) Synergy between subtherapeutic doses of cyclosporine and immunobiological manipulations in rat heart graft recipients. Transplantation 46: 122S-128S
26. Ueda H, Hancock WW, Cheung Y, Diamantstein T, Tilney N, Kupiec-Weglinski JW (1990) The mechanism of synergistic interaction between anti-interleukin 2 receptor monoclonal antibody and cyclosporine therapy in rat recipients of organ allografts. Transplantation 50: 545–550
27. Ueda H, Cheung Y, Masetti P, Diaco M, Martin M, Kupiec- Weglinski JW, Tilney NL (1991) Synergy between cyclosporine and anti-IL-2 receptor monoclonal antibodies in rats. Functional studies of heart and kidney allografts. Transplantation 52: 437–442