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The Effect of Combination Cyclosporine and CTLA4-Ig Therapy on Cardiac Allograft Survival

STEVEN F. BOLLING, M.D.,[1] HUA LIN, M.D., RU-QI WEI, M.D.,
PETER LINSLEY, PH.D., AND LAURENCE A. TURKA, M.D.

Department of Surgery (Thoracic Surgery) and Internal Medicine, University of Michigan Medical Center, Ann Arbor, Michigan 48109

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Transplant rejection requires not only T cell receptor/CD3 complex activation by foreign MHC, but also additional costimulatory signals, as T cell receptor activation alone is insufficient for induction of the immune response. The CD28 receptor on helper T cells, interacting with its ligand B7 on activated B cells or macrophages, provides this costimulus to support T cell activity. CTLA4Ig (a soluble CD28 receptor analog), binds B7 and inhibits CD28 activation. As cyclosporine (CsA) has many side effects and CTLA4Ig alone has a significant benefit upon cardiac allograft survival, we theorized that allograft survival could be improved by using CTLA4Ig with lowered dose CsA. *In vitro*, high-dose CTLA4Ig inhibited the mixed lymphocyte culture reaction (MLR) between MHC-incompatible rat strains. Furthermore, there was synergistic suppression of MLR by low-dose CTLA4Ig combined with low-dose CsA. *In vivo* studies used a cervical heterotopic transplant model. Control recipients received no immunotherapy. Experimental recipients received low-dose CsA (1.5 mg/kg/day im) × 14 days after transplant or CTLA4Ig (10, 50, or 150 μg IP × 7 days). Combination animals received both CTLA4Ig and CsA. These studies showed that low doses of CsA and CTLA4Ig were additive *in vivo*, although no additional benefit was seen when CsA was combined with high-dose CTLA4Ig. These data suggest that the combination of low-dose CsA plus CTLA4Ig may prove useful in clinical transplantation to maximize immunosuppression and minimize side effects. © 1994 Academic Press, Inc.

INTRODUCTION

Immunosuppression using cyclosporine (CsA), an inhibitor of interleukin-2 production, has made cardiac transplantation accepted therapy for end-stage cardiac failure. Cardiac transplantation offers a return to an acceptable lifestyle; however, rejection, a T cell immune response, remains as a major morbidity [1]. This antigen-specific T cell activation is initiated through the T cell receptor (TCR) [2]. Evidence has shown that T cells require two signals for activation [3] via stimulation through the TCR and by costimulation from a T cell surface receptor. In fact, stimulation of the TCR receptor without costimulation can induce a state of T cell anergy, rendering cells unresponsive to subsequent antigenic challenge [3].

The CD28 receptor on helper T cells serves as a cell surface signal transducer. Stimulation of CD28 by its ligand B7, expressed by antigen presenting cells [4], provides costimulatory signals to TCR-activated T cells for lymphokine production and cell proliferation [5]. CTLA-4 is a gene closely related to CD28 which can also serves as a B7 ligand [6]. CTLA4Ig, a soluble recombinant protein, contains the extracellular domain of human CTLA-4 fused to a human immunoglobulin Cγ chain [6]. CTLA4Ig has a 20-fold higher affinity for B7 than CD28 [6] and acts as a competitive inhibitor of CD28 activation. CTLA4Ig binds efficiently to murine and rat B7 and inhibits B7-dependent immune responses *in vitro* [6]. *In vivo*, CTLA4Ig blocks T cell-dependent B cell antibody production and delays rejection of xenogeneic islet and allogeneic cardiac allografts [7–9].

Therefore, as CsA is the most commonly used immunosuppressive drug, but has many side effects and CTLA4Ig alone has a significant benefit upon cardiac allograft survival, we theorized that allograft survival could be improved by using CTLA4Ig with a lowered dose of CsA. This present study investigated the mechanism and potential importance of CTLA4Ig and CsA relationships with regards to immunosuppression for cardiac transplantation.

METHODS

Animals. The experiments were conducted using inbred male Lewis (LEW, RT1[l]) and Brown Norway (BN, RT1[n]) rats weighing 200 to 300 g (Harlan Sprague–

[1] To whom reprint requests should be addressed at The University of Michigan Hospitals, Section of Thoracic Surgery, 2120D Taubman Center, Box 0344, 1500 E. Medical Center Drive, Ann Arbor, MI 48109-0344.

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Dawley Inc., Walkersville, MD). LEW rats served as heterotopic cardiac allograft recipients. BN rats were used as cardiac allograft donors.

Transplantation. Adult male Brown Norway rats (300–350 g) were anesthetized and mechanically ventilated. A midline incision was made from the neck to the xyphoid. The left superior vena cava, left carotid, and left and right subclavian arteries were ligated. The right carotid artery was cannulated with a catheter for heparinization and perfusion of the donor. The right superior and inferior vena cavae and left lung were ligated and divided. The heart–lung preparation was removed while being retrograde perfused through the right carotid artery cannula. The perfusate was a modified Krebs–Ringers solution, aerated with 95% O_2 and 5% CO_2 at a perfusion pressure of 80 cm of H_2O and a temperature of 37°C. Preparation of the donor heart was completed by occlusion of the descending aorta and ligation and division of the right lung at the hilum. The donor heart was continuously perfused while awaiting and during transplantation and remained in normal sinus rhythm. Next, the recipient Lewis rat was anesthetized and a midline incision was made from the sternum to the mandible, dividing the left sternocleidomastoid for exposure. The left common carotid artery was isolated, the distal portion ligated and the proximal artery occluded. The descending aorta of the donor was anastomosed (end to side) to the recipient left carotid artery. After completion of the arterial inflow, the venous anastomosis was performed for the left pulmonary artery of the donor heart to the left external jugular vein of the recipient. Continuous perfusion of the donor heart was terminated with restoration of blood flow into the beating donor heart from the recipient. Total perfusion time was routinely 45 min. The donor heart was observed for rhythm disturbances and the anastomoses were inspected for hemostasis. This heterotopic heart transplant technique, without ischemia or reperfusion, is accomplished with >90% survival and has been reported previously [10].

Mixed lymphocyte reaction. The effect of CTLA4Ig and CsA treatment on the primary immune response was examined in one-way mixed lymphocyte culture reactions (MLR) between Brown Norway rat stimulator cells (irradiated to prevent proliferation) added to Lewis rat responder lymphocytes. Lymphocytes were isolated from cervical and axillary nodes by gentle passage of tissue through a nylon mesh. Cells were cultured in RPMI 1640 medium supplemented with 5 m*M* Hepes, penicillin (10^5 units/liter), streptomycin (100 mg/liter), 50 m*M* 2-mercaptoethanol, and 10% fetal calf serum (GIBCO). Next, 3×10^5 each of responder cells and irradiated (3000 rads, ^{137}Cs source) stimulator cells were cocultured for 4 days in 96-well flat-bottomed microtiter plates as described [7]. Proliferation, measured as DNA synthesis, was determined by adding 1 μCi of [^{3}H]-thymidine (ICN) to each well for the last 6 hr of culture. All assays were performed in quadruplicate. The effect of CTLA4Ig and CsA treatment were examined by performing the MLR inhibiting assay in the presence of increasing levels of CTLA4Ig or CsA or both (Table 2).

Protocols. Brown Norway recipients of Lewis allografts were randomly divided into seven treatment groups, following transplant: control animals who received no immunosuppression; animals who received low-dose CsA only (1.5 mg/kg/day × 14 days, 1/10 the dose of CsA required to permanently prevent rejection in this model) [4, 9, 10]; animals who received CTLA4Ig (10 μg ip × 7 days); animals who received CTLA4Ig (50 μg ip × 7 days); animals who received CTLA4Ig (150 μg ip × 7 days); combination animals who received CTLA4Ig (50 μg ip × 7 days) and low-dose CsA; and combination animals who received CTLA4Ig (150 μg ip × 7 days) and low-dose CsA. No animal received any other immunosuppression. The animals were recovered and monitored on a daily basis with palpation and electrocardiograms to determine the continued viability of the grafted heart. Rejection was determined by the absence of palpable contractions in the transplanted heart and confirmed by autopsy and histologic examination. Student's *t* test analysis or analysis of variance was used to compare differences between experimental groups. Differences were considered to be statistically significant at a confidence limit of 95% ($P < 0.05$). Animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Pub. No. 80-23, revised 1978).

RESULTS

Actuarial allograft survival data are presented in Table 1. Heart transplant rejection as assessed by mean survival time to cessation of cardiac contraction was significantly delayed by administration of CsA (mean graft survival days = 14.8 ± 2.4 for CsA vs 6.8 ± 0.6 in controls). While CTLA4Ig treatment at the 10 μg/day dose (7.6 ± 1.4 days) had little effect, CTLA4Ig treatment at the 50 and 150 μg/day dose did prolong survival to 13.2 ± 2.6 days and 26.2 ± 0.6 days, respectively ($P < 0.05$ vs control). Furthermore, CTLA4Ig treatment at the 50 μg/day dose in combination with CsA significantly and synergistically prolonged survival vs controls or CsA alone to 23.0 ± 2.9 days. However, CTLA4Ig treatment at the 150 μg/day dose in combination with CsA did not demonstrate any further prolongation of allograft survival (27.2 ± 1.5 days).

Primary MLRs of Lewis splenocytes vs Brown Norway splenocytes were performed in the presence of increasing concentrations of CsA and/or CTLA4Ig. *In vi-*

TABLE 1

Allograft Survival Data of the Effect of CTLA4Ig Treatment and CsA upon Cardiac Allograft

	n	Graft survival times (days)
Control	10	6.8 ± 0.6
CsA alone	6	14.8 ± 2.6
CTLA4Ig (10)	3	7.6 ± 1.4
CTLA4Ig (50)	5	13.2 ± 2.6
CTLA4Ig (150)	5	26.2 ± 0.6*
CTLA4Ig (50) with CsA	3	23.0 ± 2.9*
CTLA4Ig (150) with CsA	5	27.2 ± 1.5*

Note. Control group recipients received no immunosuppression. Experimental recipients received low-dose CsA (1.5 mg/kg/day im) × 14 days after transplant or CTLA4Ig (10, 50, or 150 μg ip × 7 days). Combination animals received both CTLA4Ig and CsA. Rejection was defined as a lack of contractions in the transplant. Graft survival values are means ± SD. * $P < 0.0001$ vs control.

tro, high-dose CTLA4Ig inhibited the MLR between these MHC-incompatible rat strains. Furthermore, there was synergistic suppression of MLR by low-dose CTLA4Ig combined with low-dose CsA. For example, LEW splenocytes cocultured with irradiated BN splenocytes had significantly less [^{3}H]thymidine incorporation in the presence of 1 ng/ml CTLA4Ig and 10 ng/ml CsA (2561 ± 859 cpm) vs either agent alone; 1 ng/ml CTLA4Ig (14,247 ± 2,925) or 10 ng/ml CsA (49,932 ± 5,625) treated Lewis splenocytes (3671 ± 349 vs 7828 ± 814 and 7934 ± 992 cpm), respectively. Complete results are shown in Table 2.

DISCUSSION

Improved immunosuppressive agents have allowed the widespread application of cardiac transplantation as therapy for end-stage organ failure. While cardiac transplantation offers prolonged survival, symptomatic relief, and return to an acceptable lifestyle, this effort is limited by a shortage of compatible organs, transplant rejection, and side effects of immunosuppression. Organ transplant rejection, a T cell-dependent process, occurs when the host immune system reacts against foreign MHC antigens. Because of this, therapy has lead to either specific suppression of T cell-mediated immune responses using drugs such as cyclosporin A or by specific deletion of alloreactive cells. However, evidence suggests that long-term organ graft tolerance may not always be from deletion or inhibition of alloreactive T cell function but rather with induction of specific unresponsiveness of host T cells alloreactive for donor MHC antigens [3].

During the initiation of a cell-mediated immune response to allografts, the initial activation of quiescent T cells is mediated through activation of the antigen-specific T cell receptor by engagement of foreign MHC by an allogenic antigen-presenting cell [2]. This activation leads to tyrosine kinase activity and phosphorylation of phospholipase Cγ1, breaking down membrane lipids into inositol triphosphate and diacylglycerol. Inositol triphosphate leads to the elevation of intercytoplasmic free calcium levels, while diacylglycerol activates protein kinase C. The elevation of intracellular free calcium and the activation of protein kinase C serve as second messengers that lead to many effects on the metabolism and gene expression of the responder T cell [11].

The major advance in transplantation biology and immunosuppression in the last 20 years was the discovery of CsA. In contrast to previous immunosuppressive regimens, CsA in a relatively specific manner inhibits the consequences of TCR/CD3-mediated signal transduction by blocking events that occur as a result of the rise in intracellular free calcium. CsA binds to its cytoplasmic receptor, cyclophilin, and this CsA–cyclophilin complex leads to binding and inactivation of calcineurin B, a major cellular phosphatase. CsA inhibition of T cell

TABLE 2

MLR Inhibiting Assay

	CsA (ng/ml)					
	0	1	3	10	30	100
CTLA4Ig (ng/ml)						
0	55981 ± 3294	43042 ± 29599	60981 ± 16176	49932 ± 5625	15652 ± 4701	2634 ± 632
0.01	6028 ± 18173	57299 ± 2837	49992 ± 13028	41587 ± 12156	16110 ± 2031	2079 ± 1097
0.033	58295 ± 15438	63693 ± 8511	64107 ± 4469	35759 ± 9763	9025 ± 1066	747 ± 177
0.1	35021 ± 9291	39235 ± 7010	36535 ± 7593	21967 ± 5285	4602 ± 787	521 ± 39
0.33	22952 ± 5795	22325 ± 1731	27966 ± 7297	12678 ± 1822	25611 ± 859	383 ± 64
1	14247 ± 2925	16754 ± 4241	12729 ± 121944	6894 ± 1016	1696 ± 411	494 ± 88
10	14003 ± 1571	—	—	—	—	—
100	—	—	—	—	—	—

Note. The effect of CTLA4Ig and CsA, in increasing concentrations, on the primary immune response in one-way mixed lymphocyte culture reactions between Brown Norway rat stimulator cells (irradiated to prevent proliferation) added to Lewis rat responder lymphocytes.

signal transduction results in helper T cells unable to initiate an immune response by either the production of lymphokines or by proliferation. CsA inhibits the production of IL-2, IL-3, IL-4, and interferon [12–14]. Recently, it was demonstrated that CsA effectively inhibited the proliferative response and IL-2 production induced with anti CD3, but not from anti CD28. This suggests that the CD28 pathway is CsA independent and that blocking CD28 with CTLA4Ig should synergistically enhance immunosuppression [15].

However, CsA immunosuppression has direct side effects such as opportunistic infection, malignancies, and nephrotoxicity. CsA has also been associated with pericarditis and cardiomyopathy [13, 17]. All of these CsA-related side effects are proportional to systemic dose and/or target tissue level and therefore the ability to lessen CsA dosage and yet afford effective immunosuppression, perhaps with CTLA4Ig, could lead to lessened toxic side effects and better patient outcome.

The CD28 signal transduction pathway has been identified as the second signal necessary for T cell activation [5]. The CD28 molecule, expressed on 80% of peripheral blood T cells serves as a signal transduction pathway that regulates lymphokine production in helper T cells. CD28 is a 44-kDa dimeric glycoprotein of the immunoglobulin gene superfamily. Its natural ligand B7 is expressed on activated B cells and macrophages [16]. The CD28 signal transduction pathway was identified by its ability to confer cyclosporin-independent T cell proliferation. Among the interleukins whose production is increased by CD28 stimulation are IL-2, TNF-α, GM-CSF, lymphotoxin, IL-3, and IFN-γ [5]. These TH_1 lymphokines regulate cell-mediated immune responses, and, as shown by our previous work [7] as well as that of others [9], play a role in organ graft rejection.

The CD28 T cell activation pathway is potentially an important way to intervene in allogenic immune responses, as blocking the ability of presenting B7 might prevent T cell activation and perhaps induce specific anergy. *In vitro,* CTLA4Ig blocks the binding of recombinant CD28 to B7, and in one-way mixed lymphocyte reactions between Lewis rat (RT1) responders and Brown Norway rats ($RT1^n$) stimulators CTLA4Ig was able to block proliferation in a dose-dependent fashion with virtually complete inhibition being observed at 1 μg/ml [7]. Furthermore, our laboratory has demonstrated that blocking B7 with CTLA4Ig delays acute rejection of cardiac allografts *in vivo* [7]. This effect is distinct from other immunosuppression as B7 costimulation is TCR independent [11] and not simply due to interfering with T cell adhesion. Additionally, unlike inhibition of TCR-mediated activation, CD28 activation is not dependent on intracellular calcium and therefore is cyclosporin independent [11]. However, *in vivo* the immunosuppressive effect of CTLA4Ig is not indefinite, suggesting that T cells present during the time of treatment are not rendered permanently tolerant. Understanding whether blockade of the B7/CD28 pathway can synergize with other immunosuppressive regimens in the prevention of organ graft rejection was the goal of this study. Previous *in vitro* studies have demonstrated that IL-2 can restore antibody-specific responsiveness in T cell clones tolerized by TCR activation in the absence of a costimulatory signal. Therefore, this study examined the effects of CTLA4Ig in combination with CsA, which also acts as to block IL-2 production [10, 11]. CsA may also block cytokine-induced T cell proliferation response to antigens and mitogens [12].

In this specific model of Brown Norway rat heart transplantation into Lewis rat recipients, heterotopically transplanted hearts are acutely rejected within a week when no immunosuppression is utilized [17, 18]. Previous heterotopic rat heart transplant protocols have determined that donor hearts are permanently accepted in rats receiving full-dose CsA (15 mg/kg/day for 7 days intravenously), but are rejected acutely at 7–14 days when lower doses are used [17]. However, subtherapeutic doses of CsA can be made effective when CsA is used in combination with some other type of immunologic enhancement [13, 19]. The results from our present study concur that "subtherapeutic" administration of cyclosporine is more effective when given with CTLA4Ig. These studies showed that low doses of CsA and CTLA4Ig were additive *in vivo,* although no additional benefit was seen when combined with high-dose CTLA4Ig. These data suggest that the combination of low-dose CsA plus CTLA4Ig may prove useful in clinical transplantation to maximize immunosuppression and minimize side effects, as the known side effects of CsA and the apparent role of CTLA4Ig in antigen recognition [20] make CTLA4Ig an appealing target for combined therapy. Interaction of various immunosuppressives which act by separate distinct cellular mechanisms may be optimal immunotherapy for transplantation to reduce side effects and lead to more effective transplantation.

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