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ARTICLES

LF 15–0195, A NOVEL IMMUNOSUPPRESSIVE AGENT PREVENTS REJECTION AND INDUCES OPERATIONAL TOLERANCE IN A MOUSE CARDIAC ALLOGRAFT MODEL

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Background. **LF 15–0195 (LF) is a new analogue of 15-deoxyspergualin (DSG) that is less toxic and more potent than DSG. The present study was undertaken to determine (1) the dose response of LF monotherapy, (2) its ability to induce tolerance, and (3) its interaction with cyclosporine (CsA), FK 506 (FK), and rapamycin (RAPA).**

Methods. **Varying doses of LF were administered to determine dose-dependent effects on graft survival in a C57BL/6 to BALB/c heterotopic heart allograft mouse model. Transplanting-donor and third-party skin grafts into long-term survivors were used to assess the tolerance status. CsA, FK, and RAPA were combined with LF to determine their interactive effects on graft survival.**

Results. **The efficacy and toxicity of LF was dose dependent. High-dose LF monotherapy (>2 mg/kg) induced donor-specific operational tolerance, but it was associated with high mortality. Simultaneous administration of high-dose calcineurin inhibitors (CsA FK) prevented tolerance induced by LF. In contrast, a short course of LF combined with a subtherapeutic dose of CsA FK achieved indefinite survival of C57/BL6 cardiac allografts. RAPA and LF had a synergistic effect in induction of tolerance.**

Conclusions. **The efficacy and toxicity of LF were dose dependent. A short course of LF significantly reduced the requirement of CsA or FK to prevent rejection. RAPA and LF had synergy in induction of tolerance. These data indicate that LF may be a promising agent that warrants further studies in nonhuman primate models of transplantation.**

Immune-mediated rejection continues to be a serious impediment to successful organ transplantation. Despite a better understanding of rejection mechanisms and new developments in antirejection drugs, current therapies remain to be optimized. Immunosuppression has been the cornerstone of antirejection therapy and has made successful transplantation possible. Calcineurin inhibitors such as cyclosporine A (CsA) and FK 506 (FK) are the mainstays of many immunosuppressive regimens. However, patients must remain on therapy for life and may suffer severe side effects that include increased risk of infections and certain cancers. As a result, these obstacles provide the impetus for the development of improved therapies for allograft recipients. The current goal is to induce a prolonged state of nonreactivity to the allograft while preserving an intact immune system, otherwise known as tolerance.

Deoxyspergualin (DSG) is a derivative of spergualin, an antibiotic isolated from *Bacillus laterosporus* (*1*). Although it was initially developed as an antitumor agent (*1*), its profound immunosuppressive properties were elucidated from the ability of DSG to prolong survival in rat-skin allografts (*2*) and to suppress sheep red–blood-cell induced sensitization in mice (*2*). Despite encouraging clinical results with DSG (*3*, *4*), its use has been limited because of concerns of its potential toxicity and narrow therapeutic window (*5*). Recently, a new analogue of DSG, LF 15–0195 (LF), has been developed that demonstrates increased potency in preventing allograft immune responses in vivo while minimizing host toxicity (*6*). This novel agent has recently been reported to prevent allograft rejection and to induce a donor-specific tolerance in a rat cardiac transplant model (*7*). Although these preliminary results appear promising, further studies are required to determine the future role of LF in clinical transplantation. In this mouse cardiac allograft transplant model, we attempt to determine (1) the dose-dependent efficacy and drug toxicity of LF monotherapy in preventing rejection, (2) the ability for LF monotherapy to induce donor-specific tolerance, and (3) interactive effects with CsA, FK,

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and rapamycin (RAPA) in prolonging graft survival and inducing tolerance. A comprehensive study of the mechanisms of action of LF will be described in a separate publication.

MATERIALS AND METHODS

Animals

BALB/c and C57BL/6 inbred male mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and were housed with controlled light cycles in the animal facility at the University of Western Ontario. Animals were cared for according to guidelines established by the Canadian Council on Animal Care. C57BL/6 and BALB/c mice were used as donors and recipients of heart grafts, respectively. C3H mice were used as donors of third-party skin grafts. These strain combinations were mismatched in both major and minor histocompatibility complexes.

Heart Transplantation

Food and water were not restricted for donors and recipients. Mice were anesthetized with a subcutaneous (SC) injection of atropine (0.04 mg/kg) and buprenorphine (0.05 mg/kg) as well as an intraperitoneal injection of pentobarbital (32.5 mg/kg) combined with inhalant halothane and oxygen. Heterotopic heart transplantation was performed as previously described by Corry and Russell (*8*). Briefly, the C57BL/6 donor hearts were procured through a butterfly thoracic incision. The heart graft was slowly perfused in situ with 1.0 mL of cold heparinized Ringer's lactate solution through the inferior vena cava. All major vessels were ligated except for the pulmonary artery and aorta, which were sharply transected. The ascending aorta and pulmonary artery were then anastomosed end-to-side to the BALB/c recipient by way of the infrarenal abdominal aorta and inferior vena cava using 11–0 nylon sutures, respectively. Recipients were kept on a warming blanket and under a heating lamp to recover postoperatively.

Clinical Criteria for Heart Graft Rejection

Heart-graft viability was monitored daily by direct abdominal palpation. The degree of viability was scored as A, beating strongly; B, noticeable decline in the intensity of palpation; or C, complete cessation of cardiac impulses. When cardiac impulses were no longer palpable, the graft was removed for routine histology.

Graft Histology

At necropsy, tissue samples were removed, fixed in 10% buffered formaldehyde, and embedded in paraffin. Sections were cut with a microtome and stained with hematoxylin-phloxine-saffron. Specimens were examined microscopically and graded for severity of rejection by a pathologist (BG), who was blinded with respect to knowledge of therapy and clinical outcome of each case. Criteria for graft rejection included the presence of vasculitis, infarction, lymphocytic infiltration, thrombosis, and hemorrhage. These changes were scored as 0 for no change, 1 for minimum change, 2 for mild change, 3 for moderate change, and 4 for marked change.

Skin Transplantation

Full-thickness skin grafts taken from donors (C57BL/6) and third parties (C3H) were cut into square pieces of 0.5 to 1 cm^2 and transplanted onto the back of the recipient's thorax (BALB/c) that had survived to postoperative day (POD) 100. Rejection was defined as complete necrosis of the skin.

Immunosuppression

LF 15-0195 (Laboratoires Fournier, France) was diluted with saline and administrated SC daily. CsA (Novartis, Basel, Switzerland) was diluted in saline and administered SC daily. FK 506 (Fujisawa, Deerfield, IL) was diluted in saline and administered orally (PO) once a day. RAPA (Wyeth-Ayerst Madison, NJ) was diluted in olive oil and administered daily PO. Experimental mono- and combination therapy were given at doses designed in protocols described below.

Experimental Groups

BALB/c mice receiving transplants of a C57BL/6 heterotopic cardiac allograft were randomly allocated into experimental groups that received various doses and regimens of immunosuppressive therapy. Each group included 6 to 10 animals. The end point of each dosing regimen was cessation of graft impulse or 100 PODs. Mice were observed daily and cardiac grafts monitored through direct palpation. Tolerance was assessed with full-thickness skin grafts transplanted into mice that survived to POD 100.

LF monotherapy. The amounts of LF given varied from 0 (control group), 0.2 mg/kg per day, 0.5 mg/kg per day, 1 mg/kg per day, 2 mg/kg per day, and 10 mg/kg per day from POD 0 to POD 20, administered SC.

Combination of LF and high-dose CsA/FK. CsA at 15 mg/day SC or FK 16 mg/kg per day PO was administered from POD 0 to POD 20. A combination of high-dose CsA or FK with 2 mg/kg per day of LF was administered from POD 0 to POD 20.

Combination of LF and low-dose CsA. CsA was reduced to 5 mg per day and administered daily until end point (POD 100). Combination therapy consisted of LF at concentrations of 1 mg/kg per day administered from POD 0 to POD 20 combined with CsA at a dose of 5 mg/kg per day from POD 1 to POD 100 or from POD 20 to POD 100. In the later group, CsA was not given until LF was withdrawn to avoid the potential complications caused by the concurrent administration of CsA and LF.

Combination of LF and low-dose FK. FK therapy was reduced to 8 mg/kg per day PO and given from POD 0 to POD 13. A second group of animals were given LF at 2 mg/kg per day from POD 0 to POD 7. A third experimental group received both treatments as described above.

Combination of RAPA and LF. RAPA was administered at doses of 2 mg/kg per day PO from POD 0 to POD 13. A second group of animals were administered LF at 2 mg/kg per day from POD 0 to POD 7. A third experimental group received both treatments as described above.

Statistical Analysis

The data were reported as the mean±standard errors. Allograft survival among experimental groups was compared using the rank-log test. Histologic findings were analyzed using the Mann-Whitney *U* test. Differences with *P* values less than 0.05 were considered significant.

RESULTS

The Efficacy and Toxicity of LF Were Dose Dependent

Table 1 summarizes graft survival in days and the percentage of deaths caused by drug toxicity at increasing levels of LF. Untreated allografts developed rejection on POD 7.5±0.2 whereas higher doses of LF (>1 mg/kg) significantly suppressed the onset of rejection. Higher doses were also associated with greater mortality from toxicity. It was observed that all animals developed some toxic effects at a dose of 10 mg/kg. Sixty percent of these animals suffered severe anemia, diarrhea, significant weight loss, and death. However, the cardiac allografts did not demonstrate evidence of rejection. Once LF was withdrawn on POD 20, animals that had experienced weight loss regained and maintained their normal weights (data not shown). These animals survived until they were killed on POD 100 with viable allografts. When the dose of LF was reduced to 2 and 1 mg/kg, the mortality caused by toxicity was reduced to 40% and 12.5%, respectively. Animals in these groups demonstrated minimal

TABLE 1. Efficacy and toxicity of LF 15–0195 (LF) are dose dependent[a]

Groups	Treatment	Individual survival (days)	MST±SE (days)	Death caused by toxicity (%)
1	None	7×4, 8×4	7.5±0.2	0
2	10 mg/kg/day	5[b],11[b],22[b],23[b],27[b],31[b],>100×4	51.9±13.3[c]	60
3	2 mg/kg/day	5[b],15[b],17[b],18[b],66[b],>100×6	65.7±12.8[c]	40
4	1 mg/kg/day	11[b],38[b],>100×6	81.1±12.6[c]	12.5
5	0.5 mg/kg/day	10,14,17,20×3,25,33×2	21.3±2.6[c]	0
6	0.2 mg/kg/day	11,12,13,14,14,15	13.2±0.6[c]	0

[a] Study group were treated with LF daily subcutaneously from day 0 to 20.
[b] Animals died or were killed, because of toxicity, with a beating heart.
[c] $P < 0.05$ versus group 1.

weight loss and side effects while maintaining viable cardiac allografts during the follow-up period. When the dose of LF was further reduced to 0.5 and 0.2 mg/kg, no animals succumbed to toxic effects of therapy. However, these doses failed to prevent cardiac allograft rejection and mean graft survival times were 21.3±2.6 and 13.2±0.6 days, respectively. These data indicate that the efficacy and toxicity of LF are dose dependent.

High-dose LF Monotherapy Completely Prevented Cardiac Allograft Rejection

Table 2 summarizes histologic changes of C57/BL6 cardiac allografts at necropsy. Untreated allografts developed severe vascular and cellular rejection, characterized by massive lymphocyte infiltration, vasculitis, hemorrhage, infarction, and thrombosis. High-dose LF (>2 mg/kg) monotherapy completely prevented cardiac allograft rejection, and, at POD 100, grafts demonstrated normal histology. Treatment with LF at a dose of 1 mg/kg showed evidence of subclinical rejection with lymphocytic infiltration and moderate vasculitis in the grafts. Heart allografts treated with low-dose LF (0.2 and 0.5 mg/kg) developed mild to moderate vascular and cellular rejection.

Long-Term Survivors Treated with High-Dose LF (>2 mg/kg) Developed Donor-Specific Operational Tolerance

Tolerance induction was assessed by transplanting skin grafts from donor mice and a third species (C3H) into BALB/c mice that had already accepted a C57/BL6 cardiac allograft over 100 days with greater than 2 mg/kg LF therapy. Results demonstrated that skin grafts from the donor strain were slowly rejected on POD 45±1.3, whereas skin grafts from C3H were rapidly rejected, with a mean survival time of 13±0.3 days ($P<0.01$). The rejection of second skin grafts did not cause the existing heart graft rejection, and there was no pathologic evidence of rejection in these heart grafts. These data indicate that long-term survivors treated with a short course of LF developed donor-specific operational tolerance.

High-Dose Calcineurin Inhibitors Prevented Tolerance Induced by LF

Results demonstrated that mean survival times of C57/BL6 allografts were markedly reduced to 41.5±5.9 days and 31.9±4.2 days, respectively, when high-dose CsA or FK were combined with LF. In comparison, LF monotherapy (2 mg/kg per day for 20 days) prolonged graft survival to 65.7±12.8 days, and 50% of them developed donor-specific tolerance. Notably, none of the allografts developed tolerance when LF was combined with high-dose of CsA or FK (Table 3). These data indicate that the use of high-dose calcineurin inhibitors mitigates tolerance induction by LF.

Short Course of LF Combined with Subtherapeutic-Dose Calcineurin Inhibitors Achieved Indefinite Survival of C57/BL6 Cardiac Allografts

Recipients receiving C57/BL6 cardiac allografts treated with a combination of LF at doses of 1 mg/kg and CsA at 5 mg/kg from POD 0 to POD 100 daily or from POD 20 to POD 100 achieved long-term survival with no evidence of allograft rejection (Table 4). CsA at 5 mg/kg monotherapy did not prevent cardiac allograft rejection and mean graft survival time was 10.1±0.3 days. LF monotherapy at a dose of 1 mg/kg led to long-term allograft survival, but subclinical rejection was observed in these grafts (Table 5). Notably, C57/BL57 cardiac allografts treated with low-dose CsA and LF on POD 100 exhibited normal histology except for some insignificant mild lymphocytic infiltration. Similar graft survival times were achieved when a short course of LF was combined with subtherapeutic doses of FK (Table 6). Neither FK monotherapy at a dose of 8 mg/kg nor LF monotherapy at a dose of 2 mg/kg for 7 days prevented rejection or achieved long-term survival. In contrast, a combination of a short

TABLE 2. Histopathologic changes in cardiac allografts at necropsy[a]

Groups	Treatment	Lymphocyte	Vasculitis	Hemorrhage	Infarction	Thrombosis
1	Control	3.0	3.0	2.0	3.0	4.0
2	LF 10 mg/kg/day	0	0	0	0	0
3	LF 2 mg/kg/day	0	0	0	0	0
4	LF 1 mg/kg/day	2.0	1.0	0	1.0	0
5	LF 0.5 mg/kg/day	1.0	2.0	0	1.0	4.0
6	LF 0.2 mg/kg/day	2.0	2.0	0	3.0	4.0

[a] Median scores; 0, normal; 1, minimum change; 2, mild change; 3, moderate change; 4, marked change.
LF, LF 15–0195.

TABLE 3. High-dose CsA–FK has a detrimental effect on tolerance induced by LF 15–0195 (LF)

Groups	Treatment	Individual Survival (days)	MST±SE (days)	Tolerance (%)
1	LF 2 mg/kg, SC, day 0–20	5[a],15[a],17[a],18[a],66[a],>100×6	65.7±12.8	60
2	CsA 15 mg/kg, SC, day 0–20	12,13×2,14,15×2	13.7±0.5	0
3	LF 2 mg/kg, SC, day 0–20+CsA 15 mg/kg, SC, day 0–20	7[a],32[a],32,33,34,52,54,66,68	41.5±5.9	0
4	FK506 16 mg/kg, PO, day 0–20	38,41,52,56,57,60×3	53±3.1	0
5	FK506 16 mg/kg, PO, day 0–20+LF 2 mg/kg, PO day 0–20	16[a],18[a],22[a],35,36×2,45,47	31.9±4.2	0

[a] Animals died or were killed with a beating heart.
CsA, cyclosporine A; FK, FK 506; SC, subcutaneous; PO, orally MST, mean survival time; SE, standard error.

TABLE 4. Short course of LF combined with a subtherapeutic dose of CsA induces indefinite survival

Groups	Treatment	Individual survival (days)	MST±SE (days)
1	CsA 5 mg/kg/day, SC, daily	9,10×4,11×2	10.1±0.3
2	LF 1mg/kg/day, SC, 0–20 days	11[a],38[a],>100×6	81.1±12.6[b]
3	CsA 5 mg/kg/day, SC, daily, day 0–day 100+LF 1 mg/kg/day, SC, 0–20 days	6[a],12[a],35[a],>100×5	69.1±15.3[b]
4	CsA 5 mg/kg/day, daily, day 21–day 100+LF 1 mg/kg/day, SC, day 0–day 20	92,>100×7	99.0±1.0[b]

[a] Animals died or were killed with a beating heart.
[b] $P<0.05$, vs group 1.
LF, LF 15–0195; CsA, cyclosporine A; MST, Mean survival time; SE, standard error; SC, subcutaneous.

TABLE 5. Histopathologic changes of cyclosporine A (CsA) combination with LF 15–0195 (LF) in cardiac allografts at necropsy[a]

Groups	Treatment	Lymphocyte	Vasculitis	Hemorrhage	Infarction	Thrombosis
1	LF 1 mg/kg/day, 0–20 days	2.0	1.0	0	0	0
2	LF 1 mg/kg/day, 0–20 days+CsA 5 mg/kg/day, 0–100 days	2.0	0	0	0	0
3	LF 1 mg/kg/day, 0–20 days+CsA 5 mg/kg/day, 20–100 days	1.0	0	0	0	0

[a] Median scores: 0, normal; 1, minimum change; 2, mild change; 3, moderate change; 4, marked change.

TABLE 6. Short course of LF15–0195 (LF) combined with a subtherapeutic dose of FK 506 induces indefinite survival

Groups	Treatment	Individual survival (days)	MST±SE (days)
1	FK 506 8 mg/kg/day PO daily	13,14,14,15,16,18,19,21	16.3±1.0
2	LF 2 mg/kg/day SC day 0–7	24[a],29,35,45,86,89	50.3±11.8
3	FK 506 8 mg/kg/day PO daily+LF 2 mg/kg/day S.C. day 0–7	13[a],30[a],54[a],68[a],>100×4	70.6±12.5[b]

[a] Animals died or were killed, because of urine obstruction, with a beating heart.
[b] $P<0.01$ versus group 1.
PO, orally; SC, subcutaneous; MST, mean survival time; SE, standard error.

course of LF and a subtherapeutic dose of FK completely prevented rejection, and 50% of the animals in this group achieved indefinite survival of C57/BL6 cardiac allografts, although there was no statistical difference in survival between LF monotherapy and LF+FK combination therapy.

RAPA and LF Had a Synergistic Effect in Induction of Donor-Specific Tolerance

RAPA combined with LF demonstrated synergistic effects in the prolongation of graft survival (Table 7). Allografts treated with LF at a dose of 2 mg/kg for 7 days combined with RAPA at a dose of 8 mg/kg for 14 days induced indefinite survival, whereas monotherapy of either agent only prolonged allograft survival to 50.3±11.8 days for LF and 42.6±4.7 days for RAPA. Skin grafts from C3H mice were rapidly rejected by BALB/c mice with a mean survival time of 12±0.3 days, whereas skin grafts from C57/BL6 remained viable until 28.3±1.3 days ($P<0.01$). These data indicate that RAPA and LF have a synergistic effect in tolerance induction in this model.

DISCUSSION

The greatest challenge to allograft survival in clinical transplantation is rejection. Most of the currently available immunosuppressive agents such as CsA and FK are very effective at inhibiting cellular immune responses (*9*). However, they are less effective at suppressing humoral responses, which are antibody mediated and believed to play a predominant role in initiating rejection, after ABO-incompatible kidney transplantation and in presensitized recipients (*10*). CsA and FK also have side effects that may be potentially dangerous (*11*). Therefore, there is a need to search for new potent immunosuppressive agents with minimal toxici-

TABLE 7. RAPA and LF have a synergistic effect to induce tolerance in a C57/BL to BALB/c cardiac allograft model

Groups	Treatment	Individual survival (days)	MST±SE (days)
1	RAPA 2 mg/kg/day PO day 0–13	18,36,43,46,50,52,53	42.6±4.7
2	LF 2 mg/kg/day SC day 0–7	24[a],29,35,45,86,89	51.2±11.8
3	LF 2 mg/kg/day SC day 0–7+RAPA 2 mg/kg/day PO day 0–13	54[a],68[a],>100×6	90.3±6.5[b]

[a] Animals died or were killed, because of urine obstruction, with a beating heart.
[b] $P<0.05$ vs group 1 or 2.
RAPA, rapamycin; LF, LF 15–0195; PO, orally; SC, subcutaneous; MST, mean survival time; SE, standard error.

ties that have effects on the humoral arm of the immune system.

LF is a novel agent recently developed that has potential benefits for treating arthritis (*12*), autoimmune diseases such as myasthenia gravis (*13*), and organ rejection. LF is the newest modification of DSG, a synthetic analogue of the antibiotic spergualin isolated from the bacteria *Bacillus laterosporus* (*1*). It has been modified to increase stability in aqueous solution and to resist in vivo oxidative metabolism (*14*). In addition, our initial results with LF in nonhuman primates have indicated that it is more potent and less toxic than DSG in a monkey receiving a kidney allograft (*15*).

Although DSG was initially developed as an antitumor agent, subsequent studies demonstrated potent in vivo immunosuppressive activity in a variety of models. These included blockade of antibody responses (*16*), attenuation or prevention of autoimmune diseases (*17*), prolongation of transplant allograft and xenograft survival (*18*, *19*), induction of transplant tolerance (*18*), and reversal of acute allograft rejection (*20*). The biochemical mechanisms of DSG have not been fully elucidated; however, it is clear that the immunosuppressive properties of DSG are different from those of existing macrolides (CsA and FK) and antimetabolites (azathioprine and mycophenolate mofetil).

DSG is a very potent inhibitor of the humoral response. Experiments have determined DSG to be very effective at preventing allograft and xenograft rejection in both rodent and primate models (*21*). Clinically, DSG has been very useful in facilitating the acceptance of allografts in the setting of ABO-incompatible transplantation. Takahashi and his colleagues (*22*) reported on 44 patients who received ABO-incompatible renal transplants that were treated with DSG and other immunosuppressive agents. They noted excellent results, with 83% 1-year and 80% 3-year graft survival rates. In another trial that examined patients with renal transplants, DSG was effective at reversing acute rejection episodes 77.5% of the time (*20*).

Unfortunately, DSG was known to induce severe gastrointestinal side effects in canines, and the long-term use of the drug was usually lethal (*23*). The adverse effects of DSG in human clinical trials appeared to be dose dependent and included facial numbness, nausea, loss of appetite, and headache. Leukocytopenia was also observed in more than 50% of patients in an open, multicenter, randomized, comparative clinical trial (*24*). In addition, toxic effects and a narrow therapeutic window limited the use of DSG in another trial (*5*). Attempts to remedy these toxic effects and to improve immunosuppressive potency led to the development of analogues such as LF.

The immunosuppressive effects of a short 20-day course of LF in this C57/BL6 to BALB/c cardiac allograft mouse model were determined to be dose dependent. High doses greater than 2 mg/kg per day were associated with greater mortality from toxic effects. When the LF dose was reduced to 1 and 2 mg/kg, the mortality was significantly reduced. Transplant recipients treated with this range of drug doses exhibited the longest graft survival times. Also, viability of donor skin grafts were significantly prolonged in these animals. Histologic examination of the cardiac grafts at necropsy demonstrated minimal evidence of rejection. Low doses of less than 1 mg/kg per day were not sufficient to prevent rejection-mediated graft loss. The precise mechanisms of LF are currently being investigated. However, because LF is chemically related to DSG, it is reasonable to believe that they share similar modes of action.

A recent study by Chiffoleau and her colleagues (*7*) demonstrated that a 20-day course of LF induced tolerance in a fully major histocompatibility complex (MHC)-mismatched heart allograft model in the rat. They observed that CD4+ spleen T cells from tolerant LF treated recipients were able to suppress in vitro proliferation of allogeneic CD4+ T cells and to transfer tolerance to second syngeneic recipients, thus demonstrating dominant suppression by regulatory cells. We have recently reported that tolerance induced by LF is mediated by the generation of suppressive dendritic cells (*25*). In addition, we have found that LF promoted the inhibition of NF-κB by mediating the hypophosphorylation of IκB by upstream IκB kinases (unpublished data). This mode of action would prevent NF-κB from dissociating from its inhibitor and would sequester it in the cytoplasm to suppress its downstream effects in the nucleus of antigen presenting cells (APCs) and lymphocytes. Furthermore, it has been demonstrated that LF promotes activation-induced cell death (AICD) of T cells, a process that has been linked to the induction of tolerance (*26*). In addition, we have recently reported that LF significantly reduces the humoral immune response in both allograft and xenograft transplant models (*27*, *28*).

Administration of high-dose calcineurin inhibitors (CsA or FK) in conjunction with LF did not prolong graft survival and may have prevented the induction of tolerance in this particular animal model. When high-dose CsA FK were combined with LF, none of the animals developed tolerance. Because tolerance induction by LF is an active immune process that necessitates complex interactions between APCs and lymphocytes with various cytokines, we believe that calcineurin inhibitors disrupt these mechanisms. The study conducted by our group and Li and his colleagues (*30*) demonstrated similar results in which CsA prevented tolerance induced by monoclonal antibody anti-CD45RB (*29*), anti-CD40L, and CTLA4-Ig (*30*). The inhibition of tolerance by these immunosuppressives in this model may be attributed to several pos-

sible mechanisms. First, calcineurin inhibitors exert their effects through binding of the cyclophilin complex to inhibit calcineurin activation triggered by T-cell receptors (*31*). This reduces several transcription factors responsible for the activation and proliferation of T cells such as interleukin (IL)-2 and interferon (IFN)-γ and contributes to their immunosuppressive effects. High doses can totally abolish IL-2 production and inhibit IFN-γ. Both of these molecules were believed to be necessary to promote tolerance (*32*). Further evidence was demonstrated that gene knockout animals for IL-2 were shown to prohibit the induction of tolerance (*33*). IL-2 is a cytokine that is important in AICD (*30*). With IL-2 blockade from CsA or FK, apoptotic factors fail to convey proper signals, leading to an accumulation of alloreactive cells (*30*). Consequently, it may be possible for surviving lymphocytes to mount a vigorous rejection response after withdrawal of immunosuppression (*30*).

Combination therapy with high doses of CsA or FK demonstrated poor results; however, subtherapeutic doses of CsA or FK combined with LF completely prevented rejection and achieved indefinite survival in the majority of animals. This interesting observation leads us to believe that low doses of CsA or FK may decrease T-cell proliferation without totally suppressing the cell–cycle-dependent expression of IL-2 and INF-γ, which are required for AICD. We believe that this phenomenon stems from two immunosuppressive agents acting on different arms of the immune response. Whereas LF exerts its effects on APCs and the humoral response, lower doses of CsA or FK may be all that is required to attain a sufficient level of T-cell immunosuppression. The clinical implications of these results would be a decrease in the effective doses of calcineurin inhibitors that are required to treat patients. With decreased drug dosages, adverse effects and toxicity will hopefully be attenuated. We believe that LF combined with a subtherapeutic dose of calcineurin inhibitors is a reasonable clinical strategy. Recently, another agent (CAMPATH-1H) has been synergistically combined with low-dose CsA to attenuate rejection. CAMPATH-1H is a monoclonal antibody against CD52, an antigen expressed on the cell surface of mature T cells and some B cells. It was observed that, in combination with low-dose CsA, the overall incidence of acute rejection in 31 patients receiving a cadaveric renal transplant was 20% (*34*).

RAPA is an antibiotic that blocks growth–factor-associated proliferative cell responses, while permitting IL-2 mediated priming for AICD. It may encourage apoptotic events through inhibition of Bcl-2/Bcl-X gene expression (*30*). The overall effects would be to decrease the rate at which alloreactive T cells replicate while increasing their susceptibility to apoptosis (*30*). In this model, RAPA, combined with LF, was demonstrated to synergistically prolong graft survival and to induce tolerance to donor-specific skin grafts. In a separate study, Li and his colleagues (*30*) reported that a combination of RAPA with MR1 and CTL4Ig prolonged the survival of skin allografts in a mouse model to greater than 120 days. Similar results were achieved when RAPA was combined with a monoclonal antibody against CD45RB (unpublished data). We believe that the effects of RAPA on cell–cycle-dependent apoptosis of alloreactive T cells may have played a significant role in graft survival. The combination of RAPA with LF improved graft survival and increased the incidence of tolerance to donor-specific skin allografts. In consideration of the fact that LF and RAPA both have powerful immunoregulatory effects on their own, it is not surprising that the combination of these two compounds have such profound effects together.

To summarize, we have determined that the effects of LF are dose dependent. Higher concentrations of LF induce donor-specific operational tolerance but are associated with high mortality because of its side effects. We have also demonstrated synergistic effects between low-dose CsA or FK with LF. In contrast, high-dose calcineurin inhibitors prevent tolerance induced by LF. RAPA has a synergistic effect with LF in induction of tolerance. However, it is well known that there is a marked difference between mice and humans in immunologic and physiologic aspects. A preclinical study with nonhuman primates is warranted to investigate this novel agent. Our initial results using LF in nonhuman primates have indicated that LF is more potent and less toxic than DSG in a monkey receiving a kidney allograft (*15*, *28*). These encouraging results support further evaluation of LF as a valuable immunotherapy in future clinical transplantation.

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GRAFT PROTECTIVE EFFECTS OF HEME OXYGENASE 1 IN MOUSE TRACHEAL TRANSPLANT-RELATED OBLITERATIVE BRONCHIOLITIS[1]

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Background. **Heme oxygenase (HO)-1, long believed to be a cytoprotective protein, has recently been identified as a graft survival gene. This study evaluates the role of HO-1 in a murine heterotopic tracheal allograft model for obliterative bronchiolitis.**

Methods. **Mice with deficient or experimentally enhanced HO-1 expression underwent subcutaneous implantation of murine tracheal isografts and allografts. Grafts were excised after 9, 16, or 21 days and evaluated by histologic examination, immunohistochemistry for HO-1 and interleukin (IL)-10 proteins, and terminal deoxynucleotide transferase-mediated dUTP nick-end labeling. To evaluate the relationships between IL-10 and HO-1, the effects of modulation of HO-1 expression on IL-10 expression were evaluated and HO-1 expression was examined in tracheal transplants from IL-10 null mice.**

Results. **Isografts demonstrated normal histology with minimal HO-1 staining, whereas allografts showed features of human airway rejection (loss of respiratory epithelium, luminal granulation tissue, lymphocytic tracheitis) with increased HO-1 staining in macrophages and mesenchymal cells. HO-1–deficient mice demonstrated a more rapid progression of the tracheal allograft injury as compared with control allografts, and this was associated with a decrease in the anti-inflammatory cytokine, IL-10. Tracheal trans-**

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