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Abstract

Pretransplant exposure to donor antigen is known to modulate recipient alloimmunity, and frequently results in sensitization. However, donor-specific transfusion (DST) can have a protolerant effect that is dependent on route, dose and coadministered immunosuppression. Rodent studies have shown in some strain combinations that portal venous (PV) DST alone can induce tolerance, and uncontrolled clinical use of PVDST has been reported. In order to determine if pretransplant PVDST has a clinically relevant salutary effect, we studied it and the influence of concomitant immunosuppression in rhesus monkeys undergoing renal allotransplantation. Animals received PVDST with unfractionated bone marrow and/or tacrolimus or sirolimus 1 week prior to transplantation. Graft survival was assessed without any posttransplant immunosuppression. PVDST alone or in combination with tacrolimus was ineffective. However, PVDST in combination with sirolimus significantly prolonged renal allograft survival to a mean of 24 days. Preoperative sirolimus alone had no effect, and peripheral DST with sirolimus prolonged graft survival in 2/4 animals, but resulted in accelerated rejection in 2/4 animals. These data demonstrate that PVDST in combination with sirolimus delays rejection in a modest but measurable way in a rigorous model. It may thus be a preferable method for donor antigen administration.

Keywords

Donor-specific transfusion; rhesus monkey; tolerance

Introduction

Alloantigen exposure typically induces a detrimental allospecific immune response. This often results in sensitization, a cellular and/or humoral effector response resulting in the accelerated rejection of transplanted allografts. However, donor-specific transfusion (DST) of allogeneic hematopoietic cells in some settings can induce an allospecific protective or regulatory response that impedes rejection. Various mechanisms have been proposed including activation-induced cell death (AICD) of effector T cells and induction of regulatory T cells (1). Indeed,
in several experimental rodent models DST combined with certain preoperative immunosuppressive therapies has been shown to induce a durable state of tolerance (2,3). As such, various DST regimens have been proposed as tools to help prevent clinical allograft rejection. These are to be distinguished from mixed chimerism approaches in that DST transfused elements are largely eliminated from the circulation rather than establishing durable chimerism; and while microchimerism has been suggested as a beneficial mechanism involved in the DST effect (4) no attempts are made, such as myeloablation, to establish engraftment of the transfused elements or macrochimerism.

A clinically relevant antirejection effect of blood transfusion has long been recognized (5), and several investigators have used variable means of DST as adjuvant maneuvers in clinical transplant trials. These have included use of blood transfusion and more recently donor bone marrow infusion with variable and at best subtle results (6-11). Importantly, the clinical use of pretransplant DST must be balanced by the risk of sensitization (12), and given that the antirejection effects are modest and somewhat stochastic, all forms of DST remain investigational. Nevertheless, several trials have been performed using donor bone marrow as a therapeutic adjunct (13). Importantly, the mechanisms facilitating a DST effect have been shown in rodents to be sensitive to concomitant use of immunosuppressive agents, with calcineurin inhibitors (CNIs) generally preventing and mammalian target of rapamycin (mTOR) inhibitors generally promoting a salutary DST effect (14,15). Thus, with improved pharmacologic immunosuppression and most significantly the introduction of CNIs (16), the DST effect has largely dissipated and the practice fallen out of favor (17,18).

In addition to the effect of immunosuppressive drugs, the route of DST has been suggested to be significant. The liver is known to play a role in tolerance to environmental antigens, likely an adaptation preventing inappropriate immune responses to gut flora and food antigens (19). Although the mechanisms are not completely elucidated, a growing body of literature implicates liver sinusoidal endothelial cells (LSECs) as playing a critical role in portal tolerance (20-24). These unique antigen-presenting cells (APCs) predispose CD4\(^+\) T cells to secrete suppressive cytokines (24). Along with Kupffer cells (25), the LSECs may create a protolerant microenvironment within the liver that can potentially alter systemic responses to alloantigens.

Expanding on these concepts, investigators have explored multiple rodent models of portal venous (PV) DST and shown it to induce lasting tolerance to subsequently placed allografts (25-27). Interestingly, PVDST, unlike peripheral DST, has been shown in mice to be aided by adjuvant CNIs, making it potentially compatible with modern CNI-based regimens (26). These results, though not validated in more clinically relevant models, have prompted limited human trials of PVDST that have suggested that PVDST decreases acute rejection episodes and immunosuppressant requirements (28-30).

We have shown that peripheral DST given as part of certain costimulation blockade-based regimens may promote protolerant effects of these therapies in a rigorous nonhuman primate (NHP) renal allograft model (31,32). However, the effects referable to DST administration have not been marked, and to date, a definitive role for DST has not been established, nor has evidence been forthcoming objectively assessing the role of CNIs, mTOR inhibitors, or route. We have therefore preformed studies to specifically evaluate the role of DST and the effect of portal administration and adjuvant immunosuppression in a clinically relevant NHP renal allograft model. We find a modest but significant effect of PVDST in combination with the mTOR inhibitor sirolimus that exceeds other approaches to DST. As such, controlled clinical studies investigating PVDST as a preferred means of donor antigen administration may be justified.
Materials and Methods

Procedures and therapeutic regimens

In order to evaluate the potential efficacy of PVDST in a rigorous clinically applicable model, outbred rhesus monkeys, aged 2–5 years, seronegative for simian immunodeficiency virus and herpes B virus, were obtained from LABS of Virginia (Yemassee, SC) or the NIH Primate Facility (Poolesville, MD) and studied in a well-characterized renal transplant model. The experiments described in this study were conducted according to the principles set forth in the ‘Guide for the Care and Use of Laboratory Animals’ Institute of Laboratory Animals Resources, National Research Council, DHHS, Pub. No. (NIH) 86–23 (19850). Donor—recipient pairs were selected based upon genetic nonidentity at MHC class II and pretransplantation reactivity in mixed lymphocyte reaction (MLR), as previously described (33). Recipient animals received donor bone marrow 7 days prior to, or in some cases at the time of, transplantation either alone, and/or in combination with a CNI, tacrolimus (Astellas Pharma US, Deerfield, IL), or an mTOR inhibitor, sirolimus (Wyeth, Philadelphia, PA), administered for the 7 days prior to transplantation. An additional i.v. dose of bone marrow was infused on day −2 to mimic the clinical protocol reported by Trivedi et al. (29,30). Recipient animals then underwent renal transplantation without any posttransplant immunosuppression. Thus, posttransplant graft survival was dependent solely on pretransplant conditioning without any posttransplant therapeutic variables. In the absence of any therapeutic manipulation, allografts are invariably terminally rejected by posttransplant day 9.

Unfractionated donor bone marrow served as the source of DST. Marrow was percutaneously harvested from the donor humerus, femur and iliac spine. Bone marrow was washed and passed through a 40 μm filter, then centrifuged over a ficoll density gradient.

In animals receiving immunosuppression, tacrolimus or sirolimus was administered orally at 1 mg/kg/day from 7 days prior to transplantation to the day of transplantation and then discontinued. Drug-level assessments were determined using the NIH clinical laboratories.

Portal venous transfusion was performed through a mini-laparotomy by injection into a colonic mesenteric vein. In control studies designed to assess the requirement for portal administration, DST was administered through peripheral veins. The experimental groups are defined in Table 1.

Renal allotransplantation was performed as previously described using standard microvascular techniques (33,34). Left nephrectomy was performed at least 3 weeks prior to transplantation, and right nephrectomy was performed at the time of transplantation. Monkeys were heparinized (100 units/kg) during organ procurement and implantation. Serum BUN, creatinine, electrolytes (Na⁺, K⁺, Ca²⁺) and hemoglobin were determined every other day until stable and then weekly thereafter. The primary endpoint studied was time to first rejection, defined as a rise in creatinine verified by renal allograft biopsy findings consistent with allograft rejection. Terminal allograft rejection was defined as a BUN >100 mg/dL or creatinine >5.0 mg/dL on two consecutive measurements and prompted euthanasia.

All experiments described in this study were conducted according to the principles set forth in ‘The Guide for the Care and Use of Laboratory Animals’ Institute of Laboratory Animals Resources, National Research Council.

MLRs

One-way MLRs were performed using freshly isolated peripheral blood mononuclear cell (PBMC) responders coincubated with irradiated PBMC stimulators (10⁵, responder to stimulator ratio 1:1), at 37°C for 5 days. Cells were procured for pretransplantation MLR prior
to the initiation of therapy, and cells procured for posttransplantation analysis were taken from animals that had completed therapy. Cells were pulsed with 1 μCi of ³H-thymidine during the final 24 h of culture and harvested onto a pressed fiberglass paper. Lymphocyte proliferation was measured as counts per minute (c.p.m) using a β-liquid scintillation counter. A stimulation index was calculated: SI = c.p.m. stimulated responder cultures/c.p.m. unstimulated cultures. One-way MLRs were performed in recipients for evidence of donor-specific hyporesponsiveness. To minimize the use of animals and adhere to the principles of refinement, reduction and replacement, donors animals eventually become recipients in this model. When donor PBMCs were used they were collected before the initiation of immunotherapy.

**Alloantibody assessment**

Donor-specific alloantibody was identified by flow cytometry of recipient serum bound to freshly isolated, nonactivated donor CD3⁺ T cells. Recipient serum was collected and frozen prior to transplant, at the time of transplant and at regular intervals following transplantation. Samples were thawed, incubated with donor PBMCs at 4°C for 30 min, washed with phosphate buffered saline (PBS), then incubated with FITC-labeled goat antimonkey IgG antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and PE-labeled anti-rhesus CD3 (Biosource, Camarillo, CA) at 4°C for 30 min then washed twice with flow cytometry buffer. Samples were analyzed on a Becton—Dickinson FACScan. Third-party and autologous PBMC served as negative controls.

**Chimerism assessment**

Chimerism was measured using short tandem repeat (STR) profiles. Genomic DNA was first isolated from peripheral blood or tissues obtained from the nonhuman primates before and after transplantation using the BioRobot EZ1 Workstation (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The DNA concentration was determined by an ultraviolet spectrophotometer (Ultriospec 2100 Pro, Amersham Biosciences, Piscataway, NJ). A total of 1 ng of DNA was used to amplify the STR alleles for TPOX and CSF1PO using STR detection kits (GenePrint Fluorescent Monoplex STR Systems, Promega, Madison, WI). Each reaction tube included 24 μL of a mixture consisting of 2.5 μL STR buffer (including, 50 mM KCl, 10 mM Tris-HCLpH 9.0, 1.5 mM MgCl₂, 0.1% Triton X-100 and 0.2 mM of each dNTP at final concentration), 2.5 μL of TPOX or CSF1PO primers, 0.05 μL Taq polymerase (5 U/μL concentration), 18.95 μL nuclease free water and 1 ng of sample DNA, covered with mineral oil. One nanogram of K562 template DNA was used as positive control, and 1μL water was used as negative control. Unless stated otherwise, all PCR reagents were purchased from Promega, Madison, WI. The reaction was run on a PTC 100 thermal cycler (MJ Research, Inc. Waltham, MA), and the steps were set up as follows: 96°C for 2 min followed by 10 cycles of 94°C for 1 min, 60°C for 1 min and 70°C for 1.5 min followed by 20 cycles of 90°C for 1 min, 60°C for 1 min, and 70°C for 1.5 min. A sample loading cocktail was prepared for capillary electrophoresis consisting of 0.5 μL fluorescent allelic ladder (CRX, 60–400 base pairs) (Promega, Madison, WI) and 24.5 μL deionized formamide (Applied Biosystems, Foster City, CA). One microliter of the amplified sample was added to the loading cocktail, and it was analyzed by the capillary electrophoresis system of an ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems). This form of analysis generally results in the generation of two PCR products from both the donor and recipient, corresponding to the presence of two alleles for the pertinent loci in each primate. The results were evaluated with Genescan software (Applied Biosystems), which generates peaks for each of the alleles amplified. The area under the curve for each peak is directly related to the amount of DNA present in the specimen. By comparing the amount of donor and recipient DNA present in a specimen, a relative amount of chimerism can be determined.
Statistics

Treatment groups were compared to one another using the Student’s *t* -test. Statistical significance was defined as a 2-tailed *p* < 0.05.

Results

Pretransplant portal venous donor bone marrow infusion as an isolated maneuver does not prolong allograft survival in rhesus monkeys

Untreated animals (*n* = 6) rejected their renal allografts within the first week and met criteria for euthanasia in a median of 6.5 days (range 5–8). These animals have been reported elsewhere (35). Two animals that were given PVDST 7 days prior to transplantation without adjuvant therapy rejected their renal allografts on day 5. Thus, some adjuvant therapy was required for a meaningful effect.

Sirolimus is a more efficacious than tacrolimus as an adjuvant immunosuppressant when paired with PVDST

Our previous experience has revealed that sirolimus may be an effective adjuvant immunosuppressant with whole blood DST (31). Similarly, mouse studies have suggested that sirolimus, but not tacrolimus, improves the antirejection effects of DST (14,36). We therefore evaluated pretransplant PVDST in the setting of concomitant sirolimus or tacrolimus therapy. Importantly, neither drug was continued after transplantation.

When tacrolimus was paired with PVDST, no consistent survival benefit could be demonstrated, but some animals had modestly prolonged survival as long as 15 days. However, when sirolimus was paired with PVDST there was a significant prolongation of survival when compared to animals receiving pretransplant PVDST alone or in combination with tacrolimus (*p* = 0.005 and 0.019, respectively, Figure 1). This survival advantage could not be attributed to pretransplant sirolimus alone as animals given sirolimus from day −7 to 0 had mean survival of 7 days, no different than untreated controls and significantly less than when PVDST was given with sirolimus (*p* = 0.009, Figure 1). Therefore, animals were given sirolimus as the adjuvant immunosuppressant along with DST for all subsequent experiments.

Pretransplant PVDST is necessary to prolong allograft survival

In order to create a more clinically applicable treatment strategy, PVDST was attempted at the time of kidney transplant followed by i.v. DST on day 5. This was paired with adjuvant sirolimus from day 0 to 7. This treatment strategy resulted in survival times of 1, 5 and 13 days after the cessation of immunosuppression on day 7 (total survival posttransplant of 8, 12 and 20 days). This was not statistically significant when compared to untreated controls and trended toward inferiority compared to pretransplant PVDST (*p* = 0.095). When the pretransplant PVDST was replaced with an i.v. DST two of four animals had prolonged survival of 22 and 25 days. However, two animals rejected their allografts in a markedly accelerated fashion on day 3 and 4, prior to the cessation of the 7-day course of sirolimus, suggesting a possible second set rejection effect using pretransplant i.v. DST versus PVDST. Although the overall survival was not significantly different when compared to PVDST-treated animals, no animals receiving PVDST developed similar accelerated rejection. There was no difference between the mean MLR stimulation indices between those animals with rapid rejection from those with modestly prolonged survival. Therefore, pretransplant portal venous alloantigen exposure appeared preferable to precondition the animals for subsequent transplant.
Decreased dose of PVDST correlates to improved allograft survival

While the NHP model is not a practical one to do multiple dose range studies, we retrospectively investigated the relationship between the number of cells given with each PVDST and survival. An overall logarithmic trend toward decreased DST dose and improved survival was noted ($R^2 = 0.32; p = 0.037$, Figure 2). This trend suggests that increasing dose of DST may in fact be inhibitory to the mechanisms promoting portal hyporesponsiveness. In fact, animals receiving greater than $1.5 \times 10^8$ cells/kg did not have survival past 14 days. Therefore, animals in subsequent treatment groups were given a maximum of $1 \times 10^8$ cell/kg PVDST.

PVDST was not associated with alloantibody formation or peripheral or hepatic chimerism at the time of transplant

Alloantibody was tested in all animals at the time of transplant, weekly and at the time of terminal rejection. At no time during the course of alloantigen exposure did the animals develop peripherally detectable alloantibody regardless of the treatment group. Animals were also tested for both peripheral and hepatic chimerism at the time of transplant and terminal rejection via the STR assay. There was no detectable peripheral or hepatic chimerism detected in any animals given PVDST.

Sirolimus monotherapy after PVDST was not adequate to prevent rejection

Animals given pretransplant PVDST and adjuvant sirolimus without posttransplant immunosuppression had significantly prolonged survival compared to untreated controls and tacrolimus-treated animals. Although tolerance was not induced, improved survival was seen suggesting that rejection could be more easily controlled in animals so treated. Therefore, we treated six animals with PVDST and sirolimus from day $-7$ to the time of terminal rejection. These animals had survival of 22, 22, 24, 48 and 89 days. One animal died at the time of transplant due to bleeding from a core needle liver biopsy and is excluded from analysis. Although this group did not have a statistically longer survival than animals that did not receive posttransplant immunosuppression, the animal with the longest survival showed donor-specific hyporesponsiveness by MLR at day 60 post transplant (Figure 3). With minimal preconditioning, animals were maintained on monotherapy immunosuppression with sirolimus for greater than 21 days.

Discussion

Alloantigen exposure prior to the time of organ transplantation has clear immunomodulatory effects. This study is the first to examine the various clinical parameters that alter the efficacy of DST in a rigorous primate renal allograft model. While the NHP model is not a practical one for testing all possible parameters and scenarios influencing the potential outcome, we were able to investigate clinically relevant aspects of route, timing, dose and adjuvant immunosuppressive therapy. In particular, therapies were dosed in keeping with previously successful mouse models and ongoing clinical studies. Our conclusions point to relatively low-dose, pretransplant portal administration of donor whole BM paired with sirolimus as being an improved strategy for the induction of hyporesponsiveness when compared to peripheral DST or DST with tacrolimus.

Several aspects of this study deserve specific mention. The effect of PVDST is not mediated by induction of even transient chimerism. Although significant doses of donor cells were given at both day $-7$ and day $-2$, no peripheral chimerism could be detected. Although we believe that the protolerant microenvironment of the liver contributes to the hyporesponsiveness generated as a result of PVDST, the persistence of donor cells within the liver was not necessary to mediate this effect. Presumably, kupffer cell and LSEC presentation of alloantigen in an indirect fashion results in systemic effects on alloreactivity. This could be due to the production
of immunosuppressive cytokine milieu, or as increasing evidence is suggesting, the allore cognition within the liver results in clonal deletion and impaired T-cell proliferation. Both of these mechanisms would be consistent with sirolimus as it is both a potentiator of AICD and prevents T-cell proliferation via blockade of IL-2 signal transduction.

These data suggest that increasing doses of alloantigen are potentially deleterious in the induction of hyporesponsiveness. The liver is constantly exposed to antigens arising from the gut and is clearly involved in the tolerance to environmental antigens. These antigens are delivered to the liver in a repetitive low-dose fashion. Thus, to best mimic the exposure of the liver to oral antigen, a strategy of low-dose, multiple or even continuous PVDST could potentially be more efficacious and deserves investigation. We speculate that a small dose of antigen, such as that in the range of physiologic antigen delivery associated with gut pathogens or food antigens, is easily controlled by, for example, LSECs, while massive antigen burden, in contrast, provides a superphysiologic stimulus leading to sensitization. This may relate to the capacity of the LSECs to process antigen. As the antigen load increases, LSECs or other intrahepatic cells involved in antigen processing may become saturated allowing more allore cognition to reach the periphery, engage with peripheral lymphocytes in secondary lymphoid tissues and result in sensitization.

The basis for prolongation of allograft survival in this model would be best studied in a model more conducive to mechanistic analysis. That being said, we believe that DST causes beneficial alloactivation prior to transplant. Terminally differentiated alloreactive memory cells may be more prone to AICD. The ideal situation would be the specific reduction of this population to a level incapable of causing allograft rejection without altering the precursor frequency of nonalloreactive populations. Although the current regimen is incapable of achieving that goal, it may be a more potent manner in which to deliver alloantigen in combination with other protolerant therapies. We believe these data to be adequate to support the use of pretransplant PVDST as a route to immunosuppression minimization in small-scale pilot human trials.

We studied bone marrow as our source of DST to mimic clinical studies being undertaken and general interest in the clinical community in marrow infusion. Several experiments have investigated the role of splenocytes in mouse DST models, most notably in combination with costimulation blockade (37,38). We performed a pilot evaluation of splenocytes in combination with bone marrow and have found no significant difference in two animals. As there was no suggestion in these preliminary studies that splenocytes were significantly better than bone marrow and marrow is more clinically accessible in the living-related donor setting, we chose marrow for these experiments.

Studies in humans have suggested that ideal marrow infusion is delivered 7 days after transplantation (13). While PVDST may be efficacious infused after transplantation, there is no way to evaluate this approach in isolation as animals reject before day 7 unless substantial immunosuppression is supplied. Nevertheless, the data presented herein support an NHP study of PVDST in combination with a suitable induction regimen. They are also in keeping with prior work in a cynomolgus monkeys describing enhanced sensitization associated with donor bone marrow exposure by peripheral administration or intrathymic injection (39,40).

Certainly, there are many variables involving dose, route, timing and other factors that can be altered and perhaps optimized. Although, a NHP model may not be ideally conducive, logistically and economically, for exhaustive exploratory assessments, it remains useful to assess the clinical relevance of novel preclinical regimens. We set out to determine if PVDST had any measurable effect using a study design, dosing regimen and administration scheme that is feasible for clinical translation. This study indicates that portal antigen exposure is a
protolerant maneuver in complex outbred primate systems. Its investigation in controlled clinical trials may be warranted.

Acknowledgments

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References


Figure 1. Percentage of animals showing posttransplant rejection-free survival, by treatment group
Pretransplant portal vein administration of donor whole bone marrow (PVDST) paired with sirolimus prolonged rejection free renal allograft survival compared to pretransplant sirolimus alone (p = 0.009), PVDST alone (p = 0.005), or PVDST paired with tacrolimus (p = 0.019). In all PVDST groups, whole bone marrow DST was administered on pretransplant day −7 via the portal vein and day −2 via peripheral vein, unless noted in the legend. Immunosuppressive agents, sirolimus and tacrolimus, were given day −7 to day 0 unless otherwise noted in the legend.
Figure 2. The relationship between cell dose of PVDST and graft survival
Shown are the nucleated cell counts in cells infused per kilogram animal body weight versus
days of survival post transplant. The trend line is a best-fit logarithmic equation $y = -50 \ln(x) + 237$, $R^2 = 0.28$. 
Figure 3.
Donor-specific hyporesponsiveness in the longest surviving animal as measured by serial donor and third-party MLR.
### Table 1
Renal allograft rejection-free survival and treatment of all transplanted monkeys

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Rejection-free survival</th>
<th>p-Value compared to group 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. No treatment</td>
<td>5, 5, 6, 7, 7, 8</td>
<td>0.007</td>
</tr>
<tr>
<td>2. Sirolimus alone (day −7 to 0)</td>
<td>7, 7, 7</td>
<td>0.009</td>
</tr>
<tr>
<td>3. BM—DST (PV day −7 &amp; i.v. day −2), alone</td>
<td>5, 5</td>
<td>0.005</td>
</tr>
<tr>
<td>4. BM—DST (i.v. day −7 &amp; i.v. day −2), Sirolimus (days −7 to 0)</td>
<td>3, 4, 22, 25</td>
<td>0.196</td>
</tr>
<tr>
<td>5. BM—DST (PV day −7 &amp; i.v. day −2), Tacrolimus (days −7 to 0)</td>
<td>4, 6, 12, 14, 15</td>
<td>0.019</td>
</tr>
<tr>
<td>6. BM—DST (PV day 0 &amp; i.v. day 5), Sirolimus (days 0 to 7)</td>
<td>8, 12, 20</td>
<td>0.095</td>
</tr>
<tr>
<td>7. BM—DST (PV day −7 &amp; i.v. day −2), Sirolimus (day −8 to day 90)</td>
<td>22, 22, 24, 48, 89</td>
<td>0.264</td>
</tr>
<tr>
<td>8. BM—DST (PV day −7 &amp; i.v. day −2), Sirolimus (days −7 to 0)</td>
<td>14, 14, 21, 26, 28, 40</td>
<td>—</td>
</tr>
</tbody>
</table>

Rejection-free survival in days was defined as the interval between the time of transplantation and the first rejection event. The p-values determined versus treatment group 8, pretransplant PVDST/sirolimus. Groups 1–2 received no DST. For all groups, kidney transplant was performed on day 0.