Published in final edited form as:

Transplantation. 2011 September 27; 92(6): 627–633. doi:10.1097/TP.0b013e31822b9264.

Sensitized recipients exhibit accelerated but not hyperacute rejection of vascularized composite tissue allografts¹

Shengli Wu^{2,4,5,6,10}, Hong Xu^{2,5,6,7,10}, Bo Chen^{2,4,5,10}, Yujie Wen^{2,4,5,10}, Olayemi M Ikusika^{2,4,5,10}, Ashley Ocker^{2,4,10}, Hong Zhao^{2,4,10}, and Suzanne T Ildstad^{2,3,4,5,6,7,9}
Institute for Cellular Therapeutics, University of Louisville, Louisville, KY, United States, 40202

Abstract

Background—Currently, the donor-recipient matching process for vascularized composite tissue allotransplantation (VCTA) closely follows the standard practices for solid organ transplantation. Sensitization is considered a contraindication to VCTA. However, the role of sensitization in VCTA rejection is largely unstudied.

Methods—Major histocompatibility-mismatched ACI (RT1^a) donors and WF (RT1^u) recipients were used to determine if sensitization would lead to hyperacute rejection in VCTA as in other organs, such as kidneys. WF rats were presensitized to ACI antigens by skin transplantation and received heterotopic osteomyocutaneous VCTA flaps. Kidney transplants served as controls.

Results—Production of anti-donor antibody was detected in WF recipients after rejection of the ACI skin grafts. Sensitized WF rats rejected VCTA grafts from ACI rats significantly faster ($P < 0.05$) than unsensitized recipients, but not hyperacutely. Rejection in the sensitized recipients was not prevented by immunosuppression with FK506 and mycophenolate mofetil. In contrast, kidney allografts from ACI rats were hyperacutely rejected within 30 minutes by sensitized recipients. To confirm the role of antibody-mediated rejection in the sensitized recipients, serum from presensitized rats was adoptively transferred into naïve WF rats. Hyperacute rejection occurred only in transplanted kidneys but not VCTA. Histological examination of tissues from acceleratedly rejected VCTA showed dense lymphocytic infiltrates, and no antibody deposition.

Conclusions—VCTA are rejected in an accelerated fashion but not hyperacutely in the presence of allosensitization and preformed anti-donor antibody. The rejection of VCTA in sensitized recipients is mainly cell-mediated and differs mechanistically from that for renal transplants.

³Corresponding Author: Suzanne T. Ildstad, M.D., Director, Institute for Cellular Therapeutics, Jewish Hospital Distinguished Professor of Transplantation, Professor of Surgery, University of Louisville, 570 South Preston Street, Suite 404, Louisville, KY 40202-1760, Phone: (502) 852-2080, Fax: (502) 852-2079, stilds01@gwise.louisville.edu.

¹This research was supported in part by NIH RO1 HL63442, NIH RO1 DK069766, NIH RO1 HL076794; The Juvenile Diabetes Research Foundation; the W. M. Keck Foundation; The Department of Defense: Office of Naval Research; and The Department of Defense: Office of Army Research (Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the Office of Army Research. This publication was made possible by Award No. W81XWH-07-1-0185 and W81XWH-09-2-0124 from the Office of Army Research; the Commonwealth of Kentucky Research Challenge Trust Fund; the W. M. Keck Foundation; and The Jewish Hospital Foundation

²Institute for Cellular Therapeutics, University of Louisville, Louisville, KY, United States, 40202-1760, USA

⁴Participated in the performance of the research

⁵Participated in the interpretation of the data

⁶Participated in the manuscript writing

⁷Participated in research design

⁸Review all data and manuscript

⁹S. Ildstad has significant equity interest in Regenex, LLC, a start-up biotech company based on the facilitating cell technology. That is the only conflict to declare.

¹⁰No conflict of interest to declare

Keywords

sensitization; vascularized composite tissue allotransplantation; rejection

Introduction

The presence of antibodies directed against the donor has usually been considered to be an absolute contraindication to solid organ transplantation. Revascularization of a transplanted organ in the presence of preformed anti-donor antibodies may result in hyperacute rejection (1). Recent advances in the control of anti-donor antibodies using intravenous immunoglobulins (IVIg) or antihuman CD20 monoclonal antibody have allowed successful transplantation across incompatibilities of the ABO and human leukocyte antigen (HLA) systems (2). However, despite these therapies, complement-dependent Ab-mediated humoral rejection still occurs in some cases, leading to loss of the graft (3). The relevance of antibody-mediated rejection for graft survival varies depending on the organ transplanted: kidney and heart are most affected by presensitization, while liver grafts are more tolerant of ABO and HLA incompatibility (4). Patients with circulating anti-donor antibodies are considered to be “sensitized.” Sensitization can result from previous blood transfusions, pregnancies, or transplants (5). Sensitization increases the risk for bone marrow and solid organ graft rejection and causes patients to be excluded as candidates for transplantation (5).

Vascularized composite tissue allotransplantation (VCTA) is emerging as a therapeutic option for reconstruction of complex tissue defects (6,7). Major trauma to the face and extremities often leaves massive defects in soft tissue and/or bone where reconstruction with native tissue is nearly impossible. Even with cosmetic repair, the functional result is often far from satisfactory. An estimated 7 million Americans annually could benefit from VCTA (8). Currently, the donor-recipient matching process for VCTA closely follows the standard practices for renal transplantation. Thus, a positive crossmatch has been considered a contraindication to VCTA (9). However, the role of antibody-mediated rejection in VCTA has not been fully addressed. The purpose of this study was to determine the role and mechanism of sensitization in VCTA rejection. We report for the first time that VCTA are not hyperacutely rejected in the presence of allosensitization and preformed antibody but mechanistically are acutely rejected by a cellular response.

Results

Sensitization of recipient WF rats

WF rats received MHC mismatched ACI skin grafts for sensitization. All of the donor skin grafts were acutely rejected within 7 days post-transplantation. In order to define the anti-donor alloantibody responses, serum was harvested from WF recipients prior to skin transplantation and weekly thereafter for 5 weeks. To document the generation of anti-donor antibody (Ab), flow cytometry cross-match (FCXM) was used. Allogeneic skin transplantation induced the development of anti-donor Ig Abs in the recipient (Figure 1A). The levels of Ig allogeneic anti-donor Abs (MFI) at 0, 1, 2, 3, 4, and 5 weeks after skin grafting were 6.09 ± 0.34 , 113.93 ± 57.4 , 403.4 ± 54.6 , 412.3 ± 65.9 , 401.2 ± 77.64 and 395.8 ± 42.7 , respectively. Anti-donor Ab level reached its peak two weeks after skin transplantation and remain elevated up to 5 weeks of follow-up time. At week 2, increased levels of isotypes of anti-donor Ig: IgG1, IgG2a, IgG2b and IgM, were detected in sensitized recipients (MFI, 273 ± 2 , 238 ± 80 , 399 ± 34 , 124 ± 11 , respectively) (Figure 1B).

VCTA survival

VCTA was performed in various treatment groups summarized in Table 1. In naïve recipients (unsensitized recipient without immunosuppression), the first sign of VCTA rejection, erythema and edema, was observed between days 4 and 9 post-transplant and followed by hair loss, scaling, and desquamation of the skin, and mummification of the whole graft (Figure 2A). Mean survival time (MST) of this (Unsen-control) group was 9.2 ± 3.6 days. By contrast, 6 of 6 naïve rats showed long-term VCTA survival with immunosuppression of MMF and FK506 (Unsen+FK+MMF, Figure 2B). However, rejection of VCTA occurred in these long-term graft survivors in this group within 4 weeks after withdrawal of immunosuppression on day 30, 55, and 90 after VCTA.

When VCTA was performed in recipients sensitized by skin grafting (Sen-control) 5 week prior, rejection was observed between day 3 and 5 (MST 3.8 ± 0.8 days). The rejection was accelerated and significantly more rapid in Sen-control group ($P < 0.05$) compared with Unsen-controls. Moreover, the accelerated rejection in sensitized recipients could not be corrected with FK506 and MMF treatment (Sen+FK+MMF) and rejection was confirmed between day 3 and 6 (MST 4.6 ± 1.3 days). When cyclophosphamide (CyP) was added to sensitized recipients treated with FK506 and MMF (Sen+FK+MMF+CyP), VCTA survival was slightly prolonged but rejection occurred within 10 days (MST of 7.0 ± 2.0 days).

Hyperacute rejection of kidney transplants in sensitized recipients

Three sensitized WF rats received kidney transplantation (Sen+Kidney) from donor ACI rats without immunosuppression. Rejection occurred in all renal allografts within 30 minutes (Table 1). The renal grafts showed edema and cyanosis as seen in hyperacute vascular rejection (Figure 2C). No urine flow could be observed through the ureter of the donor kidney for 30 minutes after reperfusion of renal allograft. In contrast, in controls of syngeneic kidney transplantation (WF to WF), abundant urine flow was observed after unclamping and the graft maintained good renal perfusion 1 week post transplantation (Figure 2D). Histologic examination with H&E staining showed obvious interstitial hemorrhage, microthrombosis, tubular injury and glomerulitis in hyperacute rejected renal allografts (Figure 2E), while histology was normal in the naïve kidney (Figure 2F).

Donor cells were rejected hyperacutely in sensitized recipients

An *in vivo* cytotoxicity assay was performed to determine the effect of anti-donor antibody on rejection of donor cells. A total of 40×10^6 CFSE-labeled naïve donor ACI splenocytes (as target cells) and naïve recipient WF splenocytes (as internal controls) were infused into WF rats presensitized with ACI skin transplantation 5 weeks ago ($n = 4$). Sensitized rats rapidly eliminated virtually all CFSE-labeled ACI splenocytes within 3 h ($99.7 \pm 0.2\%$) (Figure 3), and the anti-donor Ab titer was 395.8 ± 42.7 (MFI) in these recipients at the mean time. The dramatically increased cytotoxicity strongly suggests that Ab-mediated killing represents one dominant barrier for alloreactivity in sensitized recipients.

Role of antibody in graft rejection

In order to define the role of antibody in VCTA rejection, naïve WF rats received adoptive transfer of serum collected from sensitized WF rats. Flow cytometry cross-match (FCXM) analysis revealed that anti-donor Ig Ab was transferred and the mean level of allo-Ab was 243.6 ± 30.4 MFI 1 day after adoptive transfer of the serum. Five adoptively transferred recipients were then transplanted with a VCTA (Serum+VCTA) from ACI donors. Rejection of the allografts occurred between days 4 and 12 (MST 7.8 ± 3.3 days) post-transplant. The VCTA rejection time in adoptive transferred recipients was significantly longer compared with that in Sen-control group ($P < 0.05$), but there was no significant difference compared

with unsensitized recipients (Unsen-controls, $P > 0.05$). When adoptively transferred recipients were transplanted with a kidney from ACI donors (Serum+kidney), hyperacute rejection occurred and the renal allografts were rejected within 30 minutes. The pattern of rejection between recipients of adoptively transferred serum and recipient sensitized with skin grafts was similar.

Histological evaluation and immunofluorescence analysis

Biopsy specimens of donor flap skin and muscle were obtained from recipients with acute and accelerate rejection (during rejection) and long-term acceptors. Histologic examination with H&E staining demonstrated a moderate infiltration of the dermal stroma by numerous mononuclear cells and destruction of architecture in the biopsies of both acutely rejected and acceleratedly rejected rat skin (Figure 4A-B). An extensive infiltration of mononuclear lymphatic cells in the biopsies of both acutely rejected and acceleratedly rejected rat muscle (Figure 4C-D) was also noted. However, skin biopsies of naïve or acceptor rats (Figure 4E-F) and muscle samples of naïve or VCTA acceptor rats (Figure 4G-H) were devoid of infiltration.

Immunofluorescence analysis of antibody deposition was performed using Alexa Fluor 647 goat anti-rat IgG. Hyperacutely rejected kidney demonstrated extensive IgG deposition (Figure 4I). In contrast, IgG staining could not be observed in acceleratedly rejected skin (Figure 4J) and muscle (Figure 4K). The quantity of integrated optical density of IgG staining in tissues of hyperacutely rejected kidney was significantly lower ($P < 0.002$) than in tissues of acceleratedly rejected skin and muscle (Figure 4L).

Discussion

Reports in the 1960s identified cases of hyperacute rejection (HAR) of kidneys from deceased donors transplanted into recipients with pre-existing antibodies against donor ABO and MHC antigens (16,17). Histologically, the major findings that are associated with HAR are neutrophil and platelet margination in glomerular and peritubular capillaries; red blood cell stasis, fibrin deposition, thrombosis within the microvasculature, acute tubular injury and variable degrees of cortical necrosis (18). Therefore, sensitization to human leukocyte antigens greatly complicates kidney, as well as other solid organ transplantation, for otherwise suitable recipients (19). Until recently, for patients who were sensitized and wanted a transplant, even if a living donor were available, a positive crossmatch was sufficient to preclude transplantation. Although some desensitization therapies have shown promise in attenuating humoral immune responses and allowing relatively safe transplantation by converting crossmatches from positive to negative (20), controversy remains as to whether attempts at overcoming the positive crossmatch offer a better balance of risk and benefit than other options, such as acceptable mismatch programs, or organ exchange programs (21,22).

VCTA refers to transplantation of multiple tissues of ectodermal and mesodermal origin (23). Currently, ABO blood group compatibility and a negative crossmatch with the recipient are necessary for VCTA donor requirements (9). However, the role of antibody-mediated rejection in VCTA remains largely unstudied. The Banff 2007 working classification of skin-containing composite tissue allograft pathology (24), came to a consensus that there is not sufficient information to draw any conclusions regarding antibody-mediated rejection in VCTA.

This study investigating whether antibody-mediate hyperacute rejection of VCTA will occur in presensitized recipients demonstrated that presensitization prior to VCTA did not result in hyperacute rejection, but instead only slightly accelerated rejection. The MST in rats

presensitized by skin transplantation was significantly shorter than that in naive control group ($P<0.05$). However, the clinical course of VCTA rejection in recipients sensitized by skin grafts was clearly different from that in kidney rejection of sensitized recipient, in which hyperacute rejection was observed and all renal allografts were rejected by sensitized WF rats within 30 minutes upon revascularization.

To demonstrate the effect of anti-donor Ab on the rejection of donor cells, *in vivo* cytotoxicity assays were performed. Sensitized rats rapidly eliminated 99.7 ± 0.2 % CFSE-labeled ACI splenocytes within 3 h. A VCTA or kidney transplantation from ACI donors to WF rats presensitized by serum adoptive transfer confirmed the role of antibody in different rejections. Again, hyperacute rejection was observed in Serum+kidney group, but not in Serum+VCTA recipients. The MST in Serum+VCTA group was not statistically different from the Unsen-control group. These results suggest that Ab-mediated rejection represents one predominant barrier for allo-kidney transplantation in sensitized recipients. The absence of hyperacute rejection in VCTA after transfer of sensitized serum may suggest that the mechanism of rejection of VCTA in sensitized recipients differs mechanistically from that for renal transplants.

To further study the mechanism of rejection in sensitized rats, H&E staining was performed on sections of VCTA or kidney tissues at the time of rejection. In renal biopsy, histological findings disclosed obvious interstitial hemorrhage, microthrombosis, tubular injury and glomerulitis, which are typical for antibody mediated hyperacute rejection. In contrast, a moderate infiltration of the dermal stroma by numerous mononuclear cells and destruction of architecture were observed in acceleratedly rejected skin biopsies, as well as an extensive infiltration of mononuclear lymphatic cells in acceleratedly rejected muscle biopsies. The histological findings in acceleratedly rejected VCTA were similar to that observed in acutely rejected tissues (Unsen-control). IgG deposition was present in hyperacutely rejected kidney tissue, but not in acceleratedly rejected rat skin and muscle, indicating that the accelerated rejection of VCTA in sensitized recipients was predominantly cellular mediated.

The histologic findings in our study suggest that the process of accelerated rejection is similar to acute rejection, but it occurs more rapidly. The mechanisms that lead to accelerated VCTA loss in presensitized recipients should differ greatly from the acute processes in unsensitized hosts, as the immune system is primed. Interestingly, sensitization is associated with a 100% risk of VCTA loss despite the immunosuppression treatment. In Sen+FK+MMF group, sensitized rats were treated with MMF and FK506, but the MST in this group was just marginally increased compared with Sen-controls ($P>0.05$). In Sen+FK+MMF+CyP group, MST in this group was statistically prolonged with addition of CyP compared with that in Sen-controls ($P<0.05$). CyP is a cytotoxic alkylating agent that primarily affects proliferating lymphocytes and has been extensively as an immunosuppressive agent (25). Several mechanisms for the efficacy of CyP in inducing allograft acceptance have been considered, with perhaps the most important being the selective effect of CyP on proliferating T cells (26). Although allografts in Sen+FK+MMF+CyP group were all eventually rejected, the finding that the MST in this group was statistically prolonged compared with group of sensitized recipients received VCTA without immunosuppression supported the mechanism of this rejection is predominantly cellular-mediated immunity, which is consistent with our histological study. Failing to control rejection in the immunosuppression treatment groups could be due to insufficient T cell suppression, and thus, novel treatment strategies need to be developed for the accelerated rejection.

In summary, presensitization prior to VCTA did not result in hyperacute rejection, but was associated with accelerated rejection. The kinetic and histological findings suggest the

mechanism of this rejection is likely predominantly cellular-mediated immunity. The role of sensitization in VCTA needs to be reevaluated, and more intensive immunosuppression treatment might be needed for the accelerated rejection.

Material and Methods

Animals

8 to 12 week old male ACI (RT1A^{abl}) and Wistar Furth (WF, RT1A^u) rats weighing 200 to 300 g were purchased from Harlan Sprague Dawley (Indianapolis, IN). All animals were housed in a specific pathogen-free facility and were treated and cared for in compliance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health.

Skin graft placement

Full thickness tail skin grafts (2 × 1cm) were transplanted as previously described (10). Rejection was defined as complete when no residual viable graft could be detected.

Flow cytometry cross-match (FCXM) assay

To evaluate the presensitization by humoral alloreactivity in allogeneic skin transplant recipients. 5×10^5 spleen cells from naïve donor were incubated with 10 μ l recipient serum samples for 25 min at 4°C. Subsequently, the samples were stained by 25-min incubation in the dark on ice with 20 μ l of 1:10 diluted FITC-conjugated polyclonal goat anti-rat Ig (BD Bioscience, San Jose, CA) Abs, and the mouse anti-rat isotype Abs of IgM, IgG1, IgG2a, and IgG2b (BD Bioscience). Levels of circulating alloantibodies were determined by FACSCalibur (BD Biosciences, Mountain View, CA) gating on the CD4⁺ or CD8⁺ T cell fractions and were reported as mean fluorescence intensity (MFI).

Experimental groups and treatment

The experimental groups were summarized in Table 1. Unsen-controls: unsensitized recipient without immunosuppression received VCTA; Unsen+FK+MMF: unsensitized recipients treated with immunosuppression consisting of mycophenolate mofetil (MMF) (15 mg/kg PO) and FK506 (1 mg/kg IP) daily starting 3 days prior to transplantation until day 30, 55, or 90 post VCTA; Sen-controls: sensitized recipients without immunosuppression received CTA; Sen+FK+MMF: sensitized recipients received FK506 + MMF + VCTA; Sen+FK+MMF+CyP: in addition to the treatment of FK506 and MMF, each sensitized VCTA recipient also received one dose cyclophosphamide (25 mg/kg IP) on day 3; Sen+kidney: sensitized recipients received kidney transplantation without immunosuppression.

Vascularized composite tissue and renal transplantation

A heterotopic osteomyocutaneous flap was performed by a technique reported previously (12). The donor flap included skin of hind limb, femur, tibia, fibula, surrounding muscles, nerves, tendons, and vessels. The flap was transplanted dorsally proximal to the hip joint of a recipient. VCTA acceptance was defined as integration of the visible skin paddle into surrounding skin, with growth of hair. Rejection was defined as loss of the skin paddle with an evolution from petechiae to eschar (13). Renal transplantation surgery was performed according to the method previously described (14). The morphological changes of donor kidney and urine flow were observed for 30 min after kidney reperfusion for evaluating hyperacute rejection.

***In vivo* cytotoxicity assay**

Bone marrow cells (BMC) from ACI/WF rats were isolated. Target (ACI) and internal control (WF) BMC ($100 \times 10^6/\text{ml}$) were incubated with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Leiden, The Netherlands) at final concentration of $4.0 \mu\text{M}$ or $0.2 \mu\text{M}$, respectively, for 10 min. Equal volume of 10% FBS (Life Technologies, Carlsbad, CA) was added to quench the reaction. 40×10^6 cells of each were injected i.v in 0.5 ml PBS. Peripheral blood was collected at 0.5, 1, 3, and 24 h, and 3, and 7 days after cell infusion. The percentage of killing was determined by the ratio between CFSE labeled target and internal control cells (15).

Serum adoptive transfer

Five weeks after sensitization by skin transplantation from ACI rats, recipient WF rats were euthanized and serums were harvested. Naïve WF rats were injected intravenously with sensitized WF rat serum at a dose of 1.5 ml per animal 1 day before receiving VCTA (Serum+VCTA) or kidney transplantation (Serum+Kidney) from ACI donors to exclude the influence of sensitized T cells in rejection (Table 1). Before transplantation, serum was collected from recipient WF rats to document the level of anti-donor antibody.

Histological evaluation

Following the VCTA, rats were inspected daily and skin and muscle biopsies were taken at the time of rejection. Kidney biopsies were performed on animals receiving a renal transplantation 4 h post-reperfusion. Samples were alcohol-dehydrated, paraffin embedded, and sectioned to a mean thickness of $5 \mu\text{m}$. The slides were stained with hematoxylin and eosin (H&E) and observed under a light microscope.

For detection of rat IgG, direct immunofluorescence was performed using Alexa Fluor 647 goat anti-rat IgG (1: 100, Invitrogen) for 1 h on sections ($5 \mu\text{m}$) of snap-frozen skin, muscle, and kidney. DAPI is used to stain DNA. Mouse pancreas was used as negative control. Photographs were taken using Leica SP5 Confocal Microscope (Leica Microsystems, Heidelberg, Germany). Quantitative assessment of IgG staining was analyzed by ImageJ software v1.45c (NIH, Bethesda, Maryland).

Statistical analyses

An unpaired student *t*-test was used for the statistical analysis. Statistical significance was given for *P* values <0.05 . Data was shown as a mean $\pm$ standard error.

Acknowledgments

The authors thank Dr. Haval Shirwan for review of the manuscript and helpful comments; Carolyn DeLautre for manuscript preparation; and the staff of the animal facility at the University of Louisville for outstanding animal care.

Abbreviations

CFSE	carboxyfluorescein diacetate succinimidyl ester
CyP	cyclophosphamide
FCXM	flow cytometry cross-match
HLA	human leukocyte antigen
MFI	mean fluorescence intensity

MMF	mycophenolate mofetil
MST	mean survival time
VCTA	vascularized composite tissue allotransplantation
WF	Wistar Furth

Reference List

1. Rowshani AT, Bemelman FJ, Lardy NM, Ten Berge IJ. Humoral immunity in renal transplantation: clinical significance and therapeutic approach. *Clin Transplant*. 2008; 22:689–699. [PubMed: 18673372]
2. Li R, Chen G, Guo H, et al. Prolonged cardiac allograft survival in presensitized rats after a high activity Yunnan-cobra venom factor therapy. *Transplant Proc*. 2006; 38:3263–3265. [PubMed: 17175243]
3. Loupy A, Suberbielle-Boissel C, Zuber J, et al. Combined posttransplant prophylactic IVIg/anti-CD 20/plasmapheresis in kidney recipients with preformed donor-specific antibodies: a pilot study. *Transplantation*. 2010; 89:1403–1410. [PubMed: 20386362]
4. Warner PR, Nester TA. ABO-incompatible solid-organ transplantation. *Am J Clin Pathol*. 2006; 125 (Suppl):S87–S94. [PubMed: 16830960]
5. Qian Z, Lee CY, Murata K, et al. Antibody and complement mediated injury in transplants following sensitization by allogeneic blood transfusion. *Transplantation*. 2006; 82:857–864. [PubMed: 17038898]
6. Wu S, Xu H, Ravindra K, Ildstad ST. Composite Tissue Allotransplantation: Past, Present and Future -The History and Expanding Applications of CTA as a New Frontier in Transplantation. *Transplant Proc*. 2009; 41:463–465. [PubMed: 19328904]
7. Ravindra KV, Wu S, McKinney M, Xu H, Ildstad ST. Composite tissue allotransplantation: current challenges. *Transplant Proc*. 2009; 41:3519–3528. [PubMed: 19917338]
8. Gander B, Brown CS, Vasilic D, et al. Composite tissue allotransplantation of the hand and face: a new frontier in transplant and reconstructive surgery. *Transpl Int*. 2006; 19:868–880. [PubMed: 17018121]
9. Ravindra KV, Buell JF, Kaufman CL, et al. Hand transplantation in the United States: experience with 3 patients. *Surgery*. 2008; 144:638–643. [PubMed: 18847649]
10. Rahhal D, Xu H, Huang WC, et al. Dissociation between peripheral blood chimerism and tolerance to hindlimb composite tissue transplants: preferential localization of chimerism in donor bone. *Transplantation*. 2009; 88:773–781. [PubMed: 19920776]
11. Yamane M, Sano Y, Nagahiro I, et al. Humoral immune responses during acute rejection in rat lung transplantation. *Transpl Immunol*. 2003; 11:31–37. [PubMed: 12727473]
12. Adamson LA, Huang W, Breidenbach WC, et al. A modified model of hindlimb osteomyocutaneous flap for the study of tolerance to composite tissue allotransplantation. *Microsurgery*. 2007; 27:630–636. [PubMed: 17868137]
13. Foster RD, Pham S, Li S, Aitouche A. Long-term acceptance of composite tissue allografts through mixed chimerism and CD28 blockade. *Transplantation*. 2003; 76:988–994. [PubMed: 14508367]
14. Chen B, Kapturczak MH, Joseph R, et al. Adeno-associated viral vector-mediated interleukin-10 prolongs allograft survival in a rat kidney transplantation model. *Am J Transplant*. 2007; 7:1112–1120. [PubMed: 17456199]
15. Xu H, Huang Y, Chilton PM, et al. Strategic nonmyeloablative conditioning: CD154:CD40 costimulatory blockade at primary bone marrow transplantation promotes engraftment for secondary bone marrow transplantation after engraftment failure. *J Immunol*. 2008; 181:6616–6624. [PubMed: 18941252]
16. Kissmeyer-Nielsen F, Olsen S, Petersen VP, Fjeldborg O. Hyperacute rejection of kidney allografts, associated with pre-existing humoral antibodies against donor cells. *Lancet*. 1966; 2:662–665. [PubMed: 4162350]

17. Williams GM, Hume DM, Hudson RP Jr, Morris PJ, Kano K, Milgrom F. "Hyperacute" renal-homograft rejection in man. *N Engl J Med*. 1968; 279:611–618. [PubMed: 4174539]
18. Racusen LC, Haas M. Antibody-mediated rejection in renal allografts: lessons from pathology. *Clin J Am Soc Nephrol*. 2006; 1:415–420. [PubMed: 17699240]
19. Magee CC. Transplantation across previously incompatible immunological barriers. *Transpl Int*. 2006; 19:87–97. [PubMed: 16441356]
20. Magee CC, Felgueiras J, Tinckam K, Malek S, Mah H, Tullius S. Renal transplantation in patients with positive lymphocytotoxicity crossmatches: one center's experience. *Transplantation*. 2008; 86:96–103. [PubMed: 18622284]
21. Stegall MD, Gloor J, Winters JL, Moore SB, Degoe S. A comparison of plasmapheresis versus high-dose IVIG desensitization in renal allograft recipients with high levels of donor specific alloantibody. *Am J Transplant*. 2006; 6:346–351. [PubMed: 16426319]
22. Jordan S. IVIG vs. plasmapheresis for desensitization: which is better? *Am J Transplant*. 2006; 6:1510–1511. [PubMed: 16827849]
23. Tobin GR, Breidenbach WC III, Pidwell DJ, Ildstad ST, Ravindra KV. Transplantation of the hand, face, and composite structures: evolution and current status. *Clin Plast Surg*. 2007; 34:271–278. [PubMed: 17418676]
24. Cendales LC, Kanitakis J, Schneeberger S, et al. The Banff 2007 working classification of skin-containing composite tissue allograft pathology. *Am J Transplant*. 2008; 8:1396–1400. [PubMed: 18444912]
25. Brode S, Cooke A. Immune-potentiating effects of the chemotherapeutic drug cyclophosphamide. *Crit Rev Immunol*. 2008; 28:109–126. [PubMed: 18540827]
26. Mayumi H, Umesue M, Nomoto K. Cyclophosphamide-induced immunological tolerance: an overview. *Immunobiology*. 1996; 195:129–139. [PubMed: 8877390]

Figure 1. Flow cytometric analysis of the Ig allo-Ab levels in presensitized rat

Donor splenocytes (5×10^5) from naive ACI rat were incubated with 10 μ l sera from sensitized WF recipients and age-matched naive controls for 25 minutes, then incubated with FITC-conjugated polyclonal goat anti-rat Ig, and mouse anti-rat IgM, IgG1, IgG2a, and IgG2b, followed by incubation with PE-conjugated anti-rat CD4 plus CD8. Levels of circulating alloantibodies were determined by FACSCalibur gating on the CD4⁺ and CD8⁺ T cells and were reported as MFI. (A) Anti-donor Ab titers were examined with staining of goat anti-rat Ig in sensitized recipients before placement of the donor skin graft (week 0) and weekly thereafter for five weeks. (B) Isotypes of Ig (IgG2a, IgG2b, IgG1 and IgM) were tested with sera collected before (week 0) and 2 weeks after skin grafting (week 2). Data are representative of 1 of 5 separate experiments. Error bars indicate SD.

Figure 2. Vascularized composite tissue grafts and kidney grafts post transplantation

A heterotopic osteomyocutaneous flap was performed from ACI donors to WF recipients. (A) In group of unsen-controls, unsensitized recipient received VCTA without immunosuppression and mummification of rejected VCTA graft was observed 7 days after transplantation. (B) In group of Unsen+FK+MMF, unsensitized recipients were treated with immunosuppression of MMF and FK506 daily starting 3 days prior to transplantation and showed indefinite VCTA survival. Pictures taken 30 days after transplantation showed flap acceptance with healthy donor hair growing. (C) WF recipients in Sen+Kidney sensitized by ACI skin transplantation received kidney transplantation from ACI rats and hyperacutely rejected the renal allografts within 30 minutes. The renal grafts clearly showed lesions of interstitial hemorrhage, edema, and cyanosis as seen in hyperacute vascularized rejection. (D) Kidney graft from naïve WF rats, serving as a syngeneic control, maintained a good renal perfusion 1 week after transplantation. The biopsies of kidney from hyperacute rejecters were embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Obvious interstitial hemorrhage, microthrombosis, tubular injury and glomerulitis were observed in this group (E). Tissues from naïve rats served as controls (F). A representative photograph taken from the sections of each group is presented.(magnification x400). Results are from two experiments.

Figure 3. Donor cells were rejected hyperacutely in sensitized recipients

A total of 40×10^6 CFSE-labeled naïve donor ACI splenocytes (as target cells) and naïve recipient WF splenocytes (as internal controls) were infused into WF rats presensitized with ACI skin transplantation 5 weeks ago. Peripheral blood was collected from individual rat at selected time points after cell infusion, as follows: 0.5 h, 1 h, 3 h, day 1, day 3, and day 7. After lysis of RBC, PBL were analyzed for CFSE expression by a FACSCalibur or LSR scanner and CellQuest software (BD Biosciences). The results are the summary of four separate experiments.

Figure 4. Histologic findings and immunofluorescence staining of IgG

The biopsies of skin or muscle of transplanted flaps from acceptors and accelerated rejecters were embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Tissues from naïve ACI rats served as controls. A representative photograph taken from the sections of each group is presented: biopsies of acutely rejected skin (A)/muscle (C); acceleratedly rejected skin (B)/muscle (D); naïve rat skin (E)/muscle (G); and acceptor skin (F)/muscle (H). Results are from two experiments (scale bar = 100 μ m). For detection of rat IgG deposition, direct immunofluorescence staining was performed using Alexa Fluor 647 goat anti-rat IgG antibody (green) (1:100) for 1 hr on sections of snap-frozen skin, muscle, and kidney. DAPI is used to stain DNA (blue). Integrated optical density analysis of IgG staining in 6 images from serial sections was performed using ImageJ software. A representative photography taken from the sections of hyperacutely rejected kidney (I), acceleratedly rejected skin (AR skin, J) and muscle (AR muscle, K) are presented (scale bar = 100 μ m). The quantity of integrated optical density of IgG staining in 6 images from serial sections was analyzed by ImageJ software n (L). Results are from two experiments.

Table 1

Experimental groups, treatment, and allograft survival

Groups	No	Sensitization	Treatment	Transplant	Rejection time	MSD
I: Unsen-control	5	No	No	VCTA	5, 8, 9, 15 days	9.2±3.6 days
II: Unsen+FK+MMF	6	No	FK506+MMF	VCTA	30 ^d , 30 ^d , 55 ^d , 90 ^d , 90 ^d days	>58.3±27.0 days ^b
III: Sen-control	6	By skin	No	VCTA	3, 3, 4, 4, 4, 5 days	3.8±0.8 days ^b
IV: Sen+FK+MMF	5	By skin	FK506+MMF	VCTA	3, 4, 4, 6, 6 days	4.6±1.3 days ^b
V: Sen+FK+MMF+CyP	4	By skin	FK506+MMF+CyP	VCTA	6, 6, 6, 10 days	7.0±2.0 days ^c
VI: Sen+kidney	3	By skin	No	Kidney	20, 25, 30 minutes	25.0±5.0 minutes ^d
VII: Serum+VCTA	5	Sensitized serum adoptive transfer	No	VCTA	4, 5, 8, 10, 12 days	7.8±3.3 days ^e
VIII: Serum+kidney	3	Sensitized serum adoptive transfer	No	Kidney	25, 30, 30 minutes	28.3±3.0 minutes ^f

^a treatment withdrawal;

^b $P<0.05$ vs group I;

^c $P<0.05$ vs group II;

^d $P<0.001$ vs group II;

^e $P<0.05$ vs group III;

^f $P<0.001$ vs group VII